

**DEVELOPMENT AND APPLICATION OF A NON-REJECTABLE COMPOSITE  
PANCREATIC ISLET ALLOGRAFT USING INDOLEAMINE 2, 3 DIOXYGENASE  
IN A DIABETIC MOUSE MODEL**

**by**

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## Abstract

Success of transplantation of pancreatic islets as a promising therapeutic method for restoring efficient regulated insulin secretion in type 1 diabetes depends on lifelong use of immunosuppressive drugs. With the goal of eliminating the necessity of systemic immunosuppressive agents after islet transplantation, in this doctoral research project we hypothesized that a novel non-rejectable islet graft through employing a local immunosuppressive factor, indoleamine 2, 3 dioxygenase (IDO) can be developed and applied. IDO is a tryptophan degrading enzyme and functions as a potent immunomodulatory factor. To address this hypothesis, we engineered a three-dimensional composite islet graft equipped with IDO expressing bystander cells as local immunosuppressive system. In this composite graft, expression of IDO in syngeneic fibroblasts provided a low tryptophan microenvironment within which T-cells could not proliferate and infiltrate islets.

Three specific aims were accomplished in this study. We first showed that local expression of IDO in syngeneic bystander fibroblasts efficiently suppressed *in vitro* proliferation of lymphocytes stimulated with allogeneic pancreatic islets. In the next phase, the impact of IDO on viability and function of mouse islets embedded within IDO-expressing fibroblast-populated collagen matrix was investigated. The result showed no reduction in islet cells viability and comparable insulin content and secretion in IDO-expressing versus control preparations. In contrast to lymphocytes, a nutrient deficiency stress-responsive pathway was not activated in islets co-cultured with IDO-expressing fibroblasts confirming the selective suppressive effect of IDO on immune versus islet cells. Furthermore, when allogeneic immune response was eliminated by using a syngeneic transplant model, IDO-expressing composite islet grafts were functional *in vivo* for up to 100 days. Finally, to

address the last specific aim, composite allogeneic islet grafts were transplanted into renal subcapsular space of streptozotocin-induced diabetic immunocompetent mice. IDO-expressing grafts survived significantly longer than controls without using any systemic immunosuppressive agent ( $41.2 \pm 1.64$  vs.  $12.9 \pm 0.73$  days,  $p < 0.001$ ). Local IDO expression evidently prevented lymphocyte infiltration into allografts and delayed alloantibody production.

The findings presented in this thesis collectively prove the potent local immunosuppressive activity of IDO in islet allografts and set the stage for development of a long-lasting non-rejectable islet allograft using stable IDO induction in bystander fibroblasts.

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## **Dedication**

This work is dedicated to:

All martyrs of my country who sacrificed their lives to bring freedom and independence to  
my nation.

My mother and father who have given their unending love and exceptional support  
throughout my life and taught me the right way to live.

And my wife, Zahra, who is a real angel for her endless love, patience and compassion.

## **Co-Authorship Statement**

The work presented in this thesis has already been published or submitted for publication as co-authored works. This is to confirm that Reza Baradar Jalili is the first author of all publications included in this thesis and that he has had the prime role in designing the experiments, performing the research, data analysis and preparation of the manuscripts of these publications.

Dr. Aziz Ghahary was the principal investigator of the research project of which this thesis was part. The main idea of making an IDO-mediated immunosuppressive shield to protect transplanted grafts was initially introduced by Dr. Ghahary. He closely supervised all experimental designs and approaches and critically reviewed all manuscripts included in this thesis. Moreover, the financial support for this thesis research project was provided through Dr. Ghahary's research grants. Other co-authors were involved in providing ideas, assisting in experiments, and reviewing the manuscripts.

# CHAPTER 1

## Introduction, Specific Aims and Research Plan

### 1.1 Overview

Type 1 diabetes mellitus (T1D) is a T-cell-mediated autoimmune disease that results in the destruction of the insulin-producing  $\beta$ -cells of the pancreatic islets of Langerhans (1). Diabetes is a serious chronic condition that causes long-term complications and significant mortality and morbidity in patients (2). At present, lifelong exogenous insulin administration by either insulin pump or multiple daily injections are considered as the standard treatment for T1D (3). However, even rigorous insulin replacement therapies are unable to adequately regulate blood glucose level and this failure results in macro and microvascular complications in many patients (4).

Transplantation of insulin-producing islets of the pancreas is a relatively new medical procedure to replace endocrine pancreatic function and has been proposed as an ideal approach to restore the efficient regulated insulin secretion that is lost in T1D (5). In fact, success in islet transplantation trials since 1990 raised the hope that islet transplantation can become a definitive treatment option for T1D (6). Unfortunately, many persistent obstacles still prevent islet transplantation from becoming the treatment of choice for T1D. For example, this procedure necessitates lifelong systemic immunosuppression. The antirejection medications have serious side-effects, including  $\beta$ -cell toxicity, hyperlipidemia, mouth ulcers, nephrotoxicity, increased risk of infection, and increased risk of development of certain malignant diseases e.g., lymphomas (7-9). As such, adverse side effects of immunosuppressive agents commonly used after transplantation are source of major concern

not only for islet cell recipients, but also for physicians, caregivers and researchers. Thus, in my doctoral research project we aimed to develop and apply a non-rejectable islet graft, using a novel approach whereby an immunosuppressive factor is locally expressed in the islet graft, creating a microenvironment in which infiltrating immune cells are unable to proliferate and destroy the engrafted islets.

## **1.2 Immunology of Graft Rejection and Role of Tryptophan Metabolism**

Rejection of allografts is a major problem in organ transplantation. The transplantation process induces many types of injury in the graft and host tissues, which can induce inflammatory responses such as MHC induction, cytokine production, chemokine expression, leukocyte infiltration, and altered gene expression in the stressed tissues (10). Although different cell types participate in the graft rejection process, only T lymphocytes appear to be absolutely required for acute rejection (11, 12). There is now compelling evidence to suggest that an immunologic response to allografts requires T-cell activation and proliferation to generate a sufficient number of infiltrating immune cells at the graft site (13). For this reason, the most effective immunosuppressive agents are those that function as anti T-cell proliferation agents such as cyclosporin and Tacrolimus.

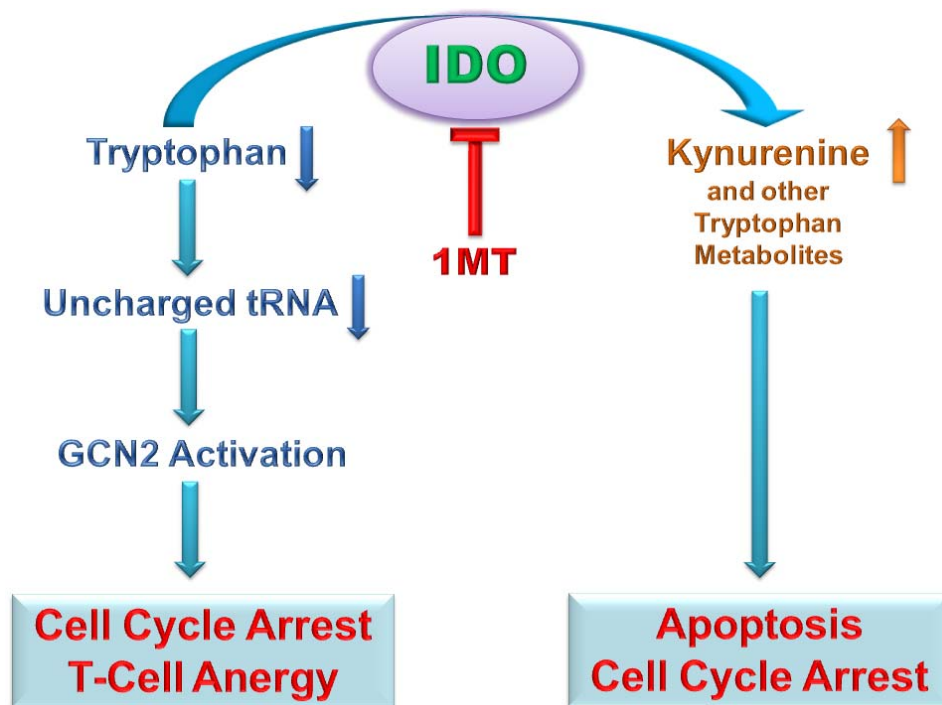
There is a balance between immune response to pathogens and tolerance to non-harmful and self antigens in the body. The mechanisms that induce tolerance are not well elucidated, but recent findings have implicated tryptophan catabolism through the kynurenine metabolic pathway as one of many mechanisms involved (14). Tryptophan is the least abundant of all essential amino acids in the human body and is required by all forms of life for protein synthesis and other important metabolic functions (15). The enzymes that break down tryptophan through kynurenine pathway are found in numerous cell types, including

cells of the immune system. Some of these enzymes are induced by immune activation, including indoleamine 2, 3-dioxygenase (IDO), the rate-limiting enzyme present mainly in macrophages and dendritic cells. It has been shown that tryptophan breakdown is necessary to maintain aspects of immune tolerance (16). Considerable evidence now supports the importance of the immunoregulatory function of IDO, including studies of mammalian pregnancy (17-19), tumor resistance (20-23), chronic infections (24-26) and autoimmune diseases (27).

Regulation of tryptophan metabolism is very critical during pregnancy. According to immunological concepts, a fetus, due to parentally acquired genes encoding antigens foreign to mother's immune system, should not survive gestation. Endogenous IDO has been implicated as one mechanism that helps maintain maternal tolerance toward the fetus as shown by the fact that mice treated early in pregnancy with 1-methyl-tryptophan (1MT), which is an inhibitor of IDO, underwent immune-mediated rejection of allogeneic concepti (17-19). These findings suggest that IDO expressing trophoblastic cells provide an immunosuppressive barrier protecting semi-allogeneic fetus from maternal T-cell immunity.

Two theories have been proposed to explain how tryptophan catabolism facilitates tolerance (Fig 1.1). One theory postulates that the downstream metabolites of tryptophan catabolism act to suppress certain immune cells, probably by pro-apoptotic mechanisms (28, 29). The other theory posits that tryptophan breakdown suppresses T-cell proliferation by dramatically reducing the supply of this critical amino acid. This was first suggested by the observation that some effects of IDO on T-cells are reversed by the addition of excess tryptophan (30, 31). Recently, the stress-responsive kinase general control nonderepressible 2 (GCN2) has been identified as a signaling molecule that enables T-cells to sense and respond

to stress conditions created by IDO (32, 33). GCN2 contains a regulatory domain that binds the uncharged form of transfer RNA. Amino acid insufficiency causes a rise in uncharged tRNA, which activates the GCN2 kinase domain and initiates downstream signaling (34). Activation of GCN2 kinase pathway, which has been termed the integrated stress response, can trigger cell cycle arrest, differentiation, compensatory adaptation, or apoptosis, depending on the cell type and the initiating stress (35, 36). It has been found that expression of IDO by antigen presenting cells (APCs) activates the GCN2 kinase pathway in responding T-cells, generating an intracellular signal that mediates key biologic effects of IDO (32).



**Figure 1.1.** Mechanisms of immunosuppression induced by IDO. IDO catalyzes the initial and rate-limiting step in the metabolism of tryptophan along the kynurenine pathway. 1-methyl-tryptophan (1MT) can function as a specific inhibitor of IDO activity. IDO enzymatic activity results in the local depletion of tryptophan and a local increase in the concentration of downstream metabolites. Tryptophan metabolites have been shown to have immunomodulatory activity, alone or in combination with the GCN2 signaling pathway. The decrease in tryptophan can cause a rise in the level of uncharged transfer RNA (tRNA) in neighboring T-cells, resulting in activation of the amino acid-sensitive GCN2 stress-kinase pathway. In turn, GCN2 signaling can cause cell cycle arrest and anergy induction in responding T-cells. The local increase in tryptophan metabolites can cause cell cycle arrest and apoptosis.



### 1.3 IDO Structure, Expression and Regulation

Mature IDO is a 42 kDa monomeric protein containing heme as its sole prosthetic group (37). Once synthesized, the IDO holoenzyme catalyzes the oxidative cleavage of the pyrrole ring of L-tryptophan to generate *N*-formyl-kynurenine that is metabolized to formic acid and the stable-end product, kynurenine. IDO has high affinity for L-tryptophan ( $K_m \sim 0.02$  mM) and therefore can rapidly catabolize it to create a local tissue microenvironment devoid of this essential amino acid (38). IDO is expressed intracellularly in a constitutive or inducible manner in different cells and tissues. IDO is constitutively expressed only in the lower gastrointestinal tract (39). Interferon-gamma (IFN- $\gamma$ ) is a potent inducer of IDO expression in placenta (40), macrophages (41), dendritic cells (42), cultured fibroblasts (43), and many cancer cell lines (44). IDO has been also detected in multiple cells that may be important to allergic inflammation including eosinophils (45), endothelial cells (46, 47), and lung epithelial cells (48).

IDO in mice and humans is encoded by a single gene, termed *Indo*, with 10 exons spread over  $\sim 1.5$  kbp of DNA located on the short arm of chromosome 8 (8p12–8p11)(49) and, as shown in murine and human dendritic cells, was found to be co-regulated by a limited number of genes (50). There is more than 60% homology between human and mouse IDO (51). Gene transcription, in general, occurs in response to inflammatory mediators, most prominently IFN- $\gamma$ , or toll-like receptor ligation (e.g. through lipopolysaccharide) (16). Within the immune system, certain types or subsets of APCs seem to be preferentially disposed to express functional IDO when challenged with proinflammatory stimuli or exposed to signals from activated T-cells. Conceptually, this ability to upregulate or downregulate IDO in response to external stimuli seems logical, given the need for APCs to

sometimes present antigens in an activating fashion and sometimes in a tolerizing fashion, depending on the context (52).

#### **1.4 IDO as a Local Immunosuppressive Factor**

As described above, there is compelling evidence to confirm that IDO possesses a potent anti-proliferative effect on T-cells both *in vitro* and *in vivo*. In view of the unique immunoregulatory function of IDO, It has been recently suggested that cells expressing IDO might contribute to the underlying mechanism of donor-specific tolerance without the use of immunosuppressive drugs. Several experiments, mostly carried out *in vitro*, corroborated the evidence that IDO activity possesses the potential to down-regulate allo-responses (42, 53). Furthermore, in an *in vivo* study, recipient mice exposed to 1-methyl-tryptophan (1MT), a specific inhibitor of IDO activity, rejected the murine liver allografts at the time of engraftment and lost spontaneous tolerogenicity usually seen after mouse liver allotransplantation (54).

Nature always provides the simplest and the best solutions to the toughest problems. As noted earlier, local expression of IDO by trophoblastic cells in the zone of contact between fetal-derived tissues and maternal immune system during murine pregnancy prevents rejection of allogeneic fetus by maternal T-cells. This very important observation inspired Dr. Aziz Ghahary and his research group to employ IDO as a local immunosuppressive factor to develop a non-rejectable allograft model. Our research group's published data provided evidence in supporting our working hypothesis that the expression of IDO in bystander fibroblasts through either IDO gene transfer or IFN- $\gamma$  treatment suppresses immune cell proliferation (55-60). Furthermore, we found that by an unknown mechanism,

only immune, but not primary skin cells are sensitive to IDO induced low tryptophan environments (55, 60).

The present thesis was evolved from our previous studies which demonstrated that IDO can indeed function as a local immunosuppressive factor in a non-rejectable skin substitute. As such, we suggested that local induction of IDO by co-transplanting genetically modified syngeneic fibroblasts will suppress the infiltrated T-cells and thereby delay or prevent allogeneic islet graft rejection without affecting the graft function and graft recipient's general immune system. These findings, thus, set the stage for the following hypothesis and objective.

## **1.5 Hypothesis, Objective and Specific Aims**

### **Hypothesis**

Local induction of immunosuppressive factor, indoleamine 2, 3-dioxygenase, a tryptophan catabolizing enzyme (delivered by collagen gel populated syngeneic fibroblasts) will protect engrafted islet cells from alloimmune rejection.

### **Objective**

Preparation and application of a composite non-rejectable allogeneic islet graft in a streptozotocin induced diabetic mouse model using adenoviral transduced IDO-expressing syngeneic fibroblasts.

This objective will be addressed through following specific aims:

- **Specific aim 1:** To test whether IDO expression in syngeneic fibroblasts can suppress allogeneic immune response to mouse pancreatic islets *in vitro*.

- **Specific Aim 2:** To evaluate the *in vitro* viability and biological function of pancreatic islets embedded within genetically modified IDO expressing fibroblast populated collagen matrix.
- **Specific Aim 3:** To prepare and transplant composite grafts consisting of allogeneic mouse islets embedded within IDO-expressing syngeneic fibroblast populated collagen gel into the renal subcapsular space of a chemically induced immunocompetent diabetic mouse and evaluate the viability, function and criteria for graft take.

## 1.6 Experimental Research Plan

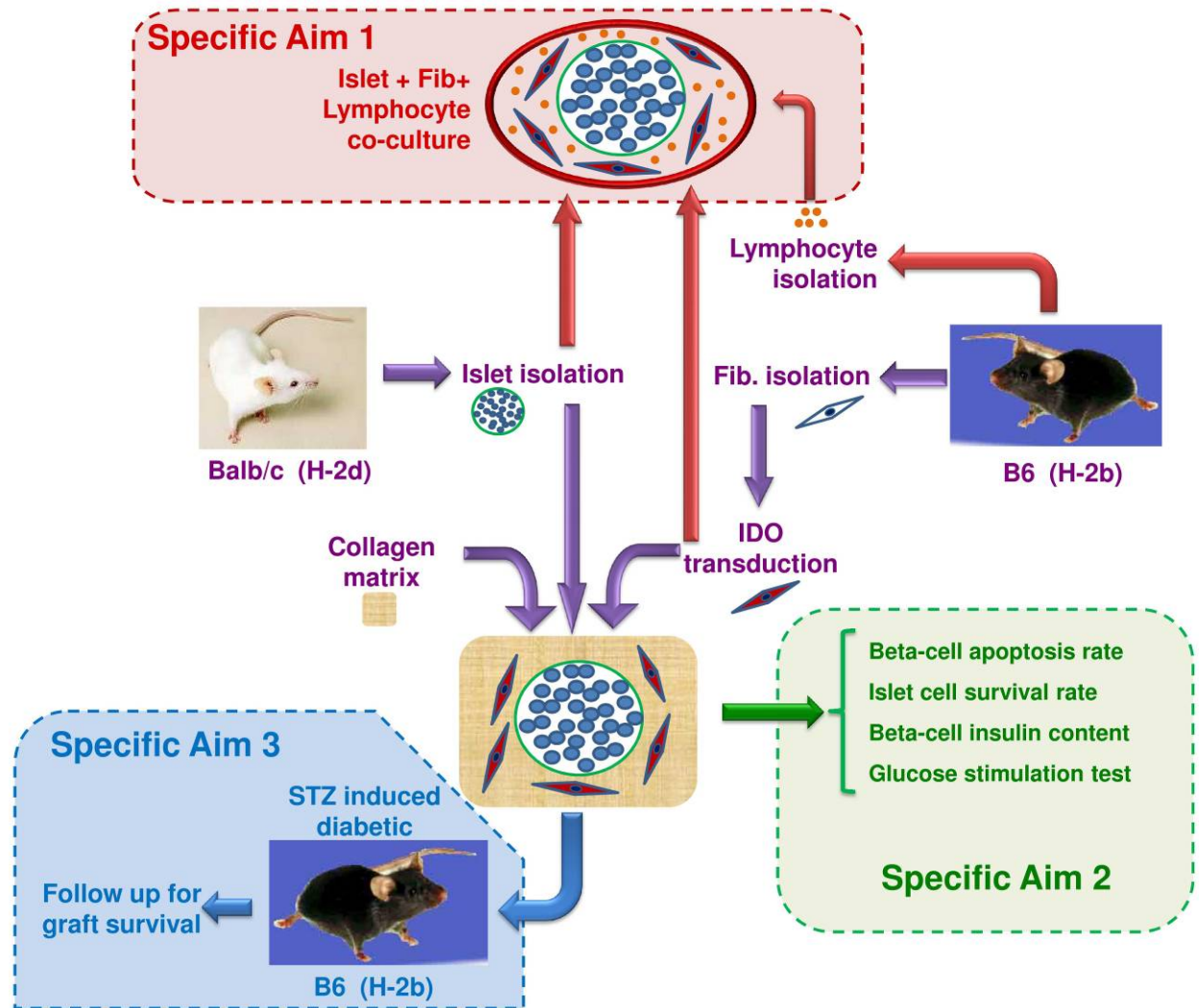
The detailed description of animals, materials, experimental methods and techniques used in this study are provided in the Material and Method sections of the following chapters. Here, a brief overview of the research plan is presented (Fig.1.2).

Fibroblast cultures were established from C57BL/6 (B6) mouse skin. Genetically modified IDO- expressing fibroblasts were then prepared using a recombinant adenoviral vector containing human IDO gene. In parallel, pancreatic islets from BALB/c mice were isolated. To address the Specific Aim 1, *in vitro* Immunosuppressive activity of IDO was evaluated in a fibroblast - islet - lymphocyte co-culture system as described in Chapter 2.

Upon validation of *in vitro* immunosuppressive function of IDO, a composite graft consisting of IDO-expressing syngeneic fibroblasts and allogeneic islets embedded within a collagen gel matrix was prepared. The reason for using collagen gel as a matrix was to construct a three dimensional cell culture system to maintain islets and fibroblast together in a way that islets are fully covered with fibroblast populated collagen gel. Collagen gel matrix has other benefits as there are several reports showing improved islet survival when cultured in a collagen gel matrix (61, 62). To address the Specific Aim 2, cell viability, apoptosis rate,

insulin content and glucose responsiveness of  $\beta$ -cells were evaluated in this composite graft (for details please see Chapter 3).

Finally, to accomplish the Specific Aim 3, the composite Balb/c mouse islet grafts were transplanted to the renal subcapsular space of streptozotocin induced diabetic B6 mice. The viability, function and criteria for graft take were then determined in the graft recipient mice as discussed in Chapter 4.



**Figure 1.2.** Experimental research plan. C57Bl/6 (B6) mouse skin fibroblasts (Fib.) were explanted and transduced to express IDO using a recombinant adenovector. Lymphocytes were also isolated from B6 mouse lymph nodes. BALB/c mouse pancreatic islets were isolated and co-cultured with B6 mouse lymphocytes and IDO-expressing fibroblasts to test IDO immunosuppressive activity *in vitro* (Specific Aim 1). IDO-expressing fibroblasts and islets were embedded within a collagen gel matrix to prepare a three-dimensional composite. Cell viability, apoptosis rate, insulin content and glucose responsiveness of  $\beta$ -cells were then evaluated in this composite co-culture (Specific Aim 2). In the next phase, the composite grafts were transplanted to streptozotocin (STZ) induced diabetic B6 mice and graft survival was assessed (Specific Aim 3).

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## **CHAPTER 2**

### **Suppression of Islet Allogeneic Immune Response by Indoleamine 2,3 Dioxygenase-Expressing Fibroblasts<sup>1</sup>**

#### **2.1 Introduction**

Type 1 diabetes is an autoimmune disorder in which insulin-secreting cells within the pancreatic islets of Langerhans are irreversibly destroyed. Transplantation of human primary islets of Langerhans has been suggested as an efficient way to control blood glucose in type 1 diabetes (1) and recent advances in pancreatic islet transplantation have brought a new hope to these patients (2). However, a series of problems remains to be solved before widespread use of this therapy can happen particularly in children. Among those obstacles is the need to use highly toxic systemic immunosuppression in islet graft recipients. One solution to this problem is to use local immunosuppressive factors. There is a model of local immunosuppressive barrier which protects semi-allogeneic fetus from being rejected by mother's immune system during pregnancy. There is convincing evidence showing that development and maintenance of the materno-fetal tolerance, at least in part, is due to tryptophan catabolism regulated by the enzyme indoleamine 2, 3-dioxygenase (IDO) which is expressed in placenta (3). A recent study by Honig et al. identified invasive extravillous trophoblast (EVT) as the location of predominant IDO expression. Since EVT are the fetal cells with the closest contact to the maternal immune system, they suggested that it is EVT which protects the fetus from rejection by downregulating local maternal T-cell responses

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<sup>1</sup> A version of this chapter has been published. Jalili RB, Rayat GR, Rajotte RV, Ghahary A: Suppression of islet allogeneic immune response by indoleamine 2,3 dioxygenase-expressing fibroblasts. J Cell Physiol. 213:137-143, 2007

through IDO expression (4). IDO is a monomeric heme-containing enzyme that catalyzes the opening of the pyrrole ring of L-tryptophan to yield N-formylkynurenine, which rapidly degrades to kynurenine (5). IDO is expressed in certain cells particularly in antigen-presenting cells (APCs) of lymphoid organs and in the placenta (6). IDO was shown to prevent rejection of the fetus during pregnancy (3) possibly by inhibiting alloreactive T-cells. Moreover, IDO expression in APCs was suggested to control autoreactive immune responses (7).

The mechanism of IDO-induced T cell suppression remains unclear. However, it has been suggested that degradation of tryptophan, the least available essential amino acid in the human body which is required for cell proliferation might be a potential way through which IDO suppresses the immune response (8). Interferon-gamma (IFN- $\gamma$ ) is a potent inducer of IDO expression in cultured fibroblasts (8), macrophages (9), dendritic cells (10) and many cancer cell lines (11). Recently published data by our group (12) showed that the expression of IDO through genetic modification (i.e. IDO protein induction by gene delivery using adenoviral vector) or IFN- $\gamma$  treatment of fibroblasts suppresses immune cell proliferation. We have been able to show that co-culturing IDO genetically modified fibroblasts with different types of immune cells, significantly increased the number of damaged bystander human PBMCs, CD4<sup>+</sup> T-cells, Jurka T-cells, THP-1 monocytes, and CD8<sup>+</sup> lymphocytes, relative to those of controls. This bystander effect proved to be due to IDO induction of a tryptophan deficient cell culture environment. In addition, this study further demonstrated that, by an unknown mechanism, only immune, but not primary skin cells are sensitive to IDO-induced low tryptophan environments (12, 13). In another series of studies our group provided compelling evidence that IFN- $\gamma$  induced IDO expression suppresses the proliferation of

immune cells co-cultured with IDO-expressing fibroblasts of an allogeneic skin substitute (14-16). Furthermore, Li et al. (17) have recently introduced a non-rejectable skin substitute model using local IDO expression.

Although IDO expression has been used initially as a local immunosuppressive factor to develop a non-rejectable skin substitute, we believe that this approach is also applicable in protecting allogeneic islet grafts. Therefore, in this study, we tested the hypothesis that local IDO expression in syngeneic fibroblasts can suppress allogeneic immune response to pancreatic islets. The effect of IDO on islet graft survival has been studied by another group (18, 19) in non-obese diabetic (NOD) mouse model which is a useful animal model for investigating the human type 1 diabetes (20). However, in these studies the investigators tried to transduce the islets directly with an IDO adenovector or a vector containing a combination of genes including IDO. Our approach in this study is different as we used a co-culture system to avoid genetic manipulation of islets. Using bystander IDO-expressing cells instead of islets is important because, any manipulation on islets including viral transduction can negatively affect islet survival and function (21-23). Therefore, by using syngeneic bystander cells as the source of IDO expression instead of genetic modification of islets, we would be able to take the advantage of IDO as a local immunosuppressive factor while avoiding any possible negative effect of those manipulations on islet survival and function.

## **2.2 Materials and Methods**

### ***In vitro* culture of fibroblasts and lymphocytes**

Skin pieces were obtained from 6-to 8-week-old male C57BL/6 (B6) mice according to a protocol approved by the institutional Animal Policy and Welfare Committee. Skin

pieces were shaved and washed three times in sterile DMEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with antibiotic-antimycotic preparation (100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B (Invitrogen). Cultures of fibroblasts were established as previously described (24). Upon reaching confluence, the cells were released using trypsin, split for subculture at a ratio of 1:4, and reseeded onto 75 cm<sup>2</sup> cell culture flasks (BD Biosciences, MA) and incubated in a humidified incubator at 37°C in an atmosphere of 5% CO<sub>2</sub>. Fibroblasts at passages three to five were used in all experiments.

Lymphocytes were isolated from peripheral lymph nodes of B6 mice by squeezing lymph node tissues between rough edges of glass slides. The cell suspension was suspended in RPMI-1640 supplemented with 10% FBS (Invitrogen) until required.

### **Isolation of pancreatic islets**

Islets were obtained from 6-to 8-week-old male BALB/c mice (The Jackson Laboratories, Bar Harbor, ME) as described by Gotoh et al. (25). Briefly, mice were anesthetized using tribromoethanol (Avertin) and pancreases were distended through the pancreatic duct with 2.5 ml of Hanks' balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD) containing 2.0 mg/ml of collagenase (Type V; Sigma Chemical Co., St. Louis, MO). The distended pancreases were then removed and incubated at 37°C for 15 min. The islets were purified by discontinuous centrifugation on Ficoll (Sigma Chemical Co.) gradients. After centrifugation, islets were handpicked and cultured in HAM's F10 medium (Sigma Chemical Co.) supplemented with 12 mM HEPES, 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 20µg/ml amphotericin B for 24 h in 95% air, 5% CO<sub>2</sub> at 37°C. Care and maintenance of all animals

was in accordance with the principles of laboratory animal care and the guidelines of institutional Animal Policy and Welfare Committee.

### **Construction of IDO adenoviral vectors**

IDO adenoviral vectors were constructed as previously described by Ghahary et al. (12). Briefly, human IDO gene was amplified by PCR then subcloned into a shuttle vector containing a GFP gene. The cloned plasmid was then homologously recombined with adenoviral plasmid in *Escherichia coli* BJ5183 by electroporation. Plasmid DNA was amplified in competent DH5 bacteria and purified by CsCl gradient in an ultracentrifuge. Adenoviral vectors carrying GFP alone or GFP plus IDO gene were used to transfect 293A package cells and amplified in large scale. The viral titer and multiplicity of infection (MOI) was determined in a 96-well flat bottom plate according to the manufacturer's (Q. Biogene, Carlsbad, CA) instructions (26).

### **Adenoviral infection of fibroblasts**

B6 mouse fibroblasts were seeded in flat bottom 12-well cell culture plates (Corning Incorporated, Corning, NY) and infected with either Ad-IDO or control viral vectors for 72 h at an MOI of 100 (Free viral particles were removed from culture medium 30 h after transfection). The expression of IDO was confirmed by: 1) monitoring GFP expression under fluorescence microscopy (Nikon, Melville, NY, HB-1010 AF); 2) the detection of IDO mRNA expression using Northern analysis; 3) the detection of IDO protein expression using western blot; and 4) measuring the level of kynurenine in conditioned medium.

### **IFN- $\gamma$ treatment of fibroblasts**

As an alternative approach to induce IDO in fibroblasts, B6 mouse fibroblasts were seeded in 12-well flat bottom cell culture plates (Corning Incorporated,) and treated with 0,



250, or 500 U/ml IFN- $\gamma$  (Sigma Chemicals, Oakville, ON, Canada) for 48 h. The expression of IDO was confirmed by: 1) the detection of IDO mRNA expression using Northern analysis; 2) the detection of IDO protein expression using western blot; and 3) measuring the level of kynurenine in conditioned medium. The rate of type I procollagen mRNA expression was also determined in IFN- $\gamma$  treated fibroblasts using Northern analysis to confirm the effectiveness of IFN- $\gamma$  treatment in fibroblasts.

### **Detection of IDO and procollagen type I mRNA by Northern analysis**

Fibroblasts were harvested and lysed with 500  $\mu$ l of 4 M guanidium isothiocyanate (GITC) and the total RNA of fibroblasts was isolated by GITC/CsCl procedure as described by Chirgwin et al. (27). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10  $\mu$ g per lane) on a 1% agarose gel containing 2.2 M formaldehyde then blotted onto nitrocellulose membrane. To control the RNA loading, quantities of 18S ribosomal RNA were compared visually by ethidium bromide fluorescence. Northern blot analysis was done as previously described (15). Quantitative analysis of mRNA expression was accomplished by densitometry of mRNA bands using Scion Image Software Version 4.0.3 (Scion Corporation, Frederick, MD).

### **Detection of IDO protein by western immunoblotting**

For detection of IDO expression, fibroblasts were harvested after 48 h of IFN- $\gamma$  treatment or 72 h post-infection and washed twice with PBS. Cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.4; 10 mM EDTA; 5 mM EGTA; 0.5% NP40; 1% Triton X-100, and protease inhibitor cocktail (Sigma). Equal amounts of total protein from each individual fibroblast culture were separated by 10% SDS-PAGE. Proteins were then transferred to a PVDF membrane (Millipore Corp., Bedford, MA) and immunoblotted with

polyclonal anti-human IDO antibody raised in rabbits by Washington Biotechnology Inc. (Baltimore, MD) at final dilution of 1:5,000. Horseradish peroxidase conjugated goat anti-rabbit IgG served as a secondary antibody for the enhanced chemiluminescence detection system (ECL; Amersham Biosciences, UK).

### **Determination of kynurenine levels in the conditioned medium**

The biological activity of IDO was evaluated by measuring the levels of tryptophan degradation product, L-kynurenine, present in conditioned medium derived from fibroblasts. The amount of L-kynurenine was measured by the previously established method (28). Briefly, proteins in conditioned medium were precipitated by trichloroacetic acid. After centrifugation, 0.5 ml of supernatant was incubated with equal volume of Ehrlich's reagent for 10 min at room temperature. Absorption of resultant solution was measured at 490 nm by spectrophotometer. The values of kynurenine in conditioned medium were calculated according to a standard curve with defined kynurenine concentration (0-20 µg/ml).

### **Co-culture of fibroblasts, islets and lymphocytes**

B6 mouse fibroblasts were either infected with Ad-IDO viral vectors for 72 h at an MOI of 100 or treated with 500 U/ml of IFN-γ for a period of 48 h. Subsequently,  $5 \times 10^4$  of infected or IFN-γ treated fibroblasts were washed and plated into 12-well flat bottom plates (Corning Incorporated), co-cultured with 100 BALB/c islets and then these co-cultures were subjected to gamma irradiation (2000 Rads). In the next stage,  $25 \times 10^4$  B6 mouse lymphocytes were added to the co-cultures in RPMI-1640 medium supplemented with 10% FBS for a period of 72 h. Specificity of IDO effect on lymphocyte proliferation was determined by addition of an IDO inhibitor, 1-methyl-DL-tryptophan (Aldrich Chemical Co.,

Milwaukee, WI), to the IDO-expressing fibroblasts culture at the final concentration of 800  $\mu$ M.

### **[3H]-thymidine incorporation assay**

To measure the rates of lymphocyte proliferation in response to stimulation with allogeneic islets, B6 mouse lymphocytes (isolated from peripheral lymph nodes of mice by squeezing lymph node tissues between rough edges of glass slides) co-cultured with syngeneic fibroblasts and allogeneic BALB/c islets and were labeled with [3H]-thymidine. Briefly, 74 KBq of [3H]-thymidine (Perkin-Elmer Life Sciences Inc., Boston, MA) was added to each ml of the conditioned medium and the cells were incubated for 16 h. After this period, lymphocytes were harvested, washed three times with PBS, dissolved in guanidium isothiocyanate, and added to scintillation fluid (Amersham Corp., Arlington Heights, IL). Radioactive counting was performed using a Beckman scintillation counter. Lymphocytes proliferation rates were reported as standardized rate (%) calculated according to the CPM of control (untreated) groups.

### **Statistical analysis**

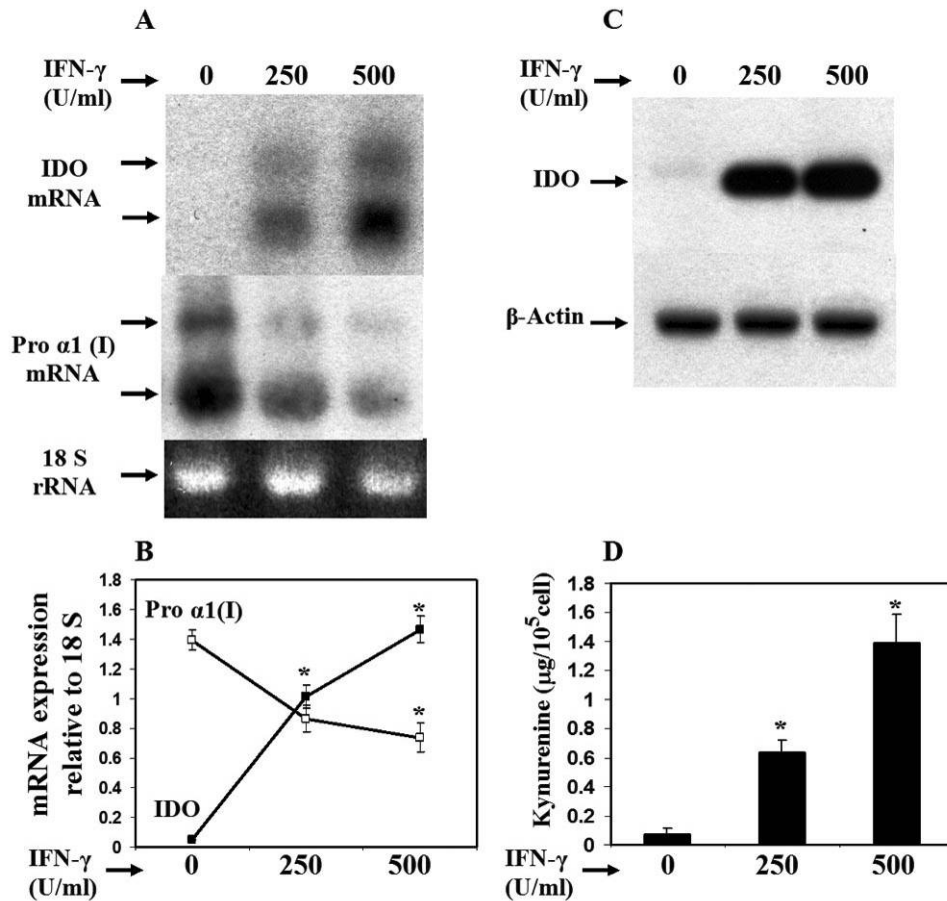
All data are reported as mean  $\pm$  SD. One-way analysis of variance with post hoc test was used to compare the kynurenine levels, densitometry results for Northern analyses and lymphocyte proliferation rates between IDO-expressing and control groups. P values less than 0.05 were considered to be significant.

## 2.3 Results

### **IFN- $\gamma$ treated mouse fibroblasts expressed functional IDO protein**

B6 mouse fibroblasts were treated with 0, 250, or 500 U/ml of IFN- $\gamma$  and after 48 h of incubation, conditioned media were collected and cells were harvested. Expression of IDO was assessed at the level of mRNA, protein and kynurenine production. Figure 2.1A shows the levels of IDO mRNA expression in fibroblasts after treatment with increasing concentrations of IFN- $\gamma$ . Northern analysis demonstrates increasing IDO mRNA expression corresponds to increasing concentration of IFN- $\gamma$  (Fig. 2.1A). A combined densitometry result of three separate experiments on the ratio of mRNA/18S expression was performed and results showed a significant dose-dependent increase in IDO mRNA expression in response to IFN- $\gamma$  treatment ( $0.05 \pm 0.02$ ,  $1.01 \pm 0.07$ , and  $1.5 \pm 0.08$ , for fibroblasts treated with 0, 250 and 500 U/ml IFN- $\gamma$ , respectively;  $P < 0.01$  relative to values of control group,  $n = 3$ ; Fig. 2.1C). In view of the fact that IFN- $\gamma$  selectively suppresses collagen production in normal fibroblasts in a time- $\gamma$  and dose-dependent manner (29), the same blots were re-probed for type I procollagen mRNA as a proof of normal response of treated fibroblasts to IFN- $\gamma$  (Fig. 2.1A). The results showed a significant dose-dependent decrease in type I procollagen mRNA production in response to IFN- $\gamma$  treatment ( $1.39 \pm 0.06$ ,  $0.86 \pm 0.08$ , and  $0.73 \pm 0.09$ , for fibroblasts treated with 0, 250 and 500 U/ml IFN- $\gamma$  respectively;  $P < 0.01$  relative to values of control group,  $n = 3$ ; Fig. 2.1C). To measure the IDO protein expression in fibroblast in response to IFN- $\gamma$  treatment, we used western blot analysis. Fibroblasts lysates were fractionated by SDS-PAGE and IDO protein was detected using a polyclonal anti-IDO antibody. The results showed a dose-dependent increase in IDO induction in response to IFN- $\gamma$  treatment (Fig. 2.1B). Finally, to show the function of induced IDO,

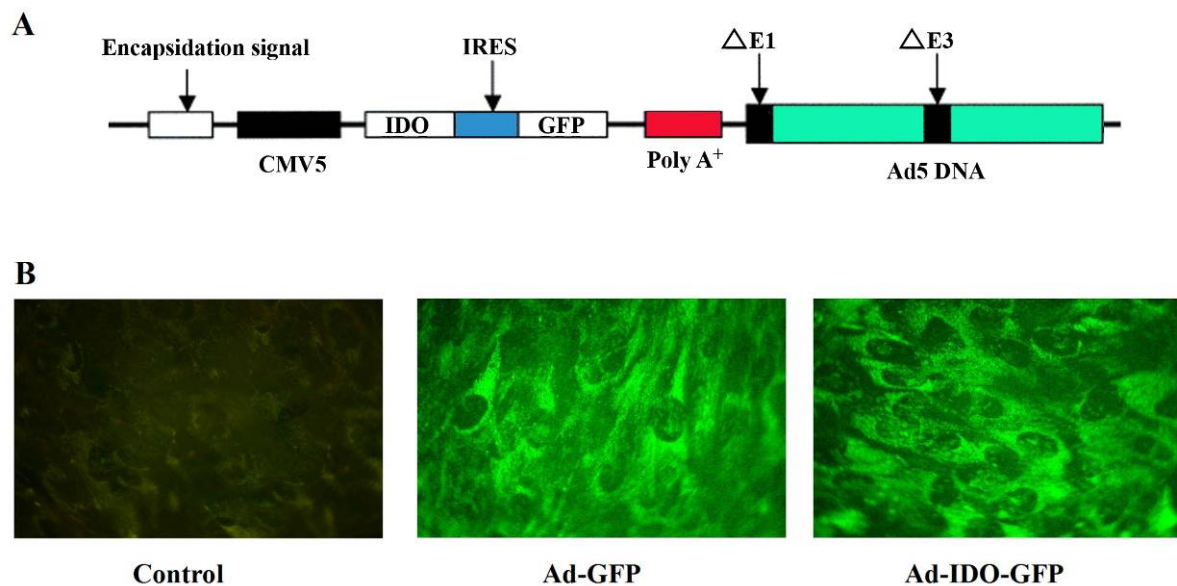
kynurenine levels were measured in conditioned media of treated fibroblasts. The average of three separate experiments showed a significant dose-dependent increase in kynurenine level ( $0.07 \pm 0.02$ ,  $0.64 \pm 0.07$ ,  $1.39 \pm 0.17$   $\mu\text{g}/10^5$  cells for fibroblasts treated with 0, 250, and 500 U/ml IFN- $\gamma$ , respectively;  $P < 0.01$  relative to values of control group,  $n = 3$ ; Fig. 2.1D).



**Figure 2.1.** IDO expression by fibroblasts in response to IFN- $\gamma$  treatment. B6 mouse fibroblasts ( $10^5$  cells per well) were treated with increasing concentrations (0, 250, or 500 U/ml) of IFN- $\gamma$  for 48 h. Conditioned media then were collected and cells were harvested. Total RNA of fibroblasts was extracted and subjected to Northern blot analysis. Part A shows IDO mRNA (two bands) and type I procollagen mRNA (two bands) expression in fibroblasts after treatment with increasing concentrations of IFN- $\gamma$ . 18S rRNA shows loading control. Part C shows the mean ratio of densities of IDO and type I procollagen mRNA bands to those of 18S rRNA in three separate experiments. The filled squares and open squares show the mean IDO and pro 1 (I) collagen mRNA/18S rRNA, respectively.\* significantly different from the control value for the same group,  $P < 0.01$ ,  $n = 3$ . In part B, western blot analysis of IDO protein expression after IFN- $\gamma$  treatment is shown. Upper arrow shows a 42 kDa band corresponding to the IDO protein. Expression of  $\beta$ -Actin protein was assessed as loading control. Part D shows mean Kynurenine levels in conditioned media of fibroblasts treated with increasing concentrations of IFN- $\gamma$ . The kynurenine production rate is reported as  $\mu\text{g}$  per  $10^5$  cultured fibroblasts.\* Significantly different from the control value,  $P < 0.01$ ,  $n = 3$ .

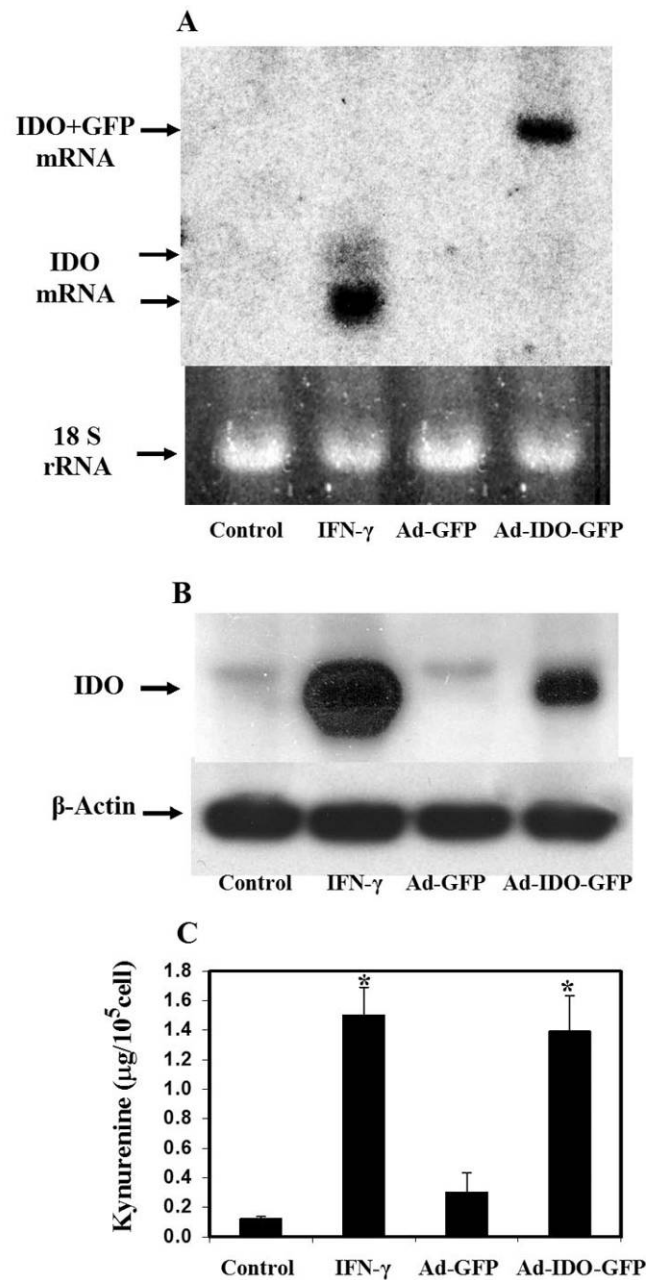
### Adenoviral vector-infected cells expressed IDO and GFP proteins

B6 mouse fibroblasts were cultured and infected with recombinant adenoviral vectors containing only GFP gene (Ad-GFP) or IDO plus GFP genes (Ad-IDO-GFP) (Fig 2.2A) as described in Methods. Microscopic evaluation of Ad-GFP (Fig. 2.2B, middle panel) and Ad-IDO-GFP (Fig. 2.2B, right panel) infected fibroblasts under UV light after 72 h showed bright green fluorescent stained cells, indicating GFP expression in these cells.



**Figure 2.2.** Schematic diagram of constructed IDO adenovector and expression of green fluorescent protein (GFP) in infected mouse fibroblasts. (A) Human IDO gene was cloned into a pAdenoVator-CMV5-IRES-GFP. (B) Expression of GFP in virus infected cells as a marker for efficiency of viral transduction. B6 mouse fibroblasts were infected with recombinant adenoviral vectors at an MOI of 100 and expression of GFP in fibroblasts was assessed under UV light after 72 h of treatment with blank medium (control), post-infection with Ad-GFP vector or Ad-IDO-GFP vector. Magnification was at 100 $\times$ .

To test the efficiency of IDO transduction in fibroblasts, B6 mouse fibroblasts were treated with medium only or IFN- $\gamma$ , or infected with Ad-GFP or Ad-IDO-GFP vectors. After 72 h post-infection, conditioned media were collected and cells were harvested. Expression of IDO in transduced fibroblasts was assessed at the levels of mRNA, protein and kynurenine production. Northern analysis showed high levels of IDO mRNA expression in IFN- $\gamma$  treated and Ad-IDO-GFP infected fibroblast but not in untreated or Ad-GFP infected fibroblast (Fig. 2.3A). Since IDO and GFP genes in our recombinant vector were linked by an internal ribosomal entry site (IRES) and expressed in a bicistronic fashion, the size of expressed mRNA in response to transduction with this vector was equal to size of IDO + GFP mRNA. At protein level, western blot analysis showed large amounts of IDO expression in IFN- $\gamma$  treated and Ad-IDO-GFP infected fibroblasts whereas there was almost no IDO expression in untreated or Ad-GFP infected fibroblasts (Fig. 2.3B). Significantly higher Kynurenine levels in conditioned media of IFN- $\gamma$  treated and Ad-IDO-GFP infected cells showed that expressed IDO was functional ( $0.12 \pm 0.02$ ,  $1.5 \pm 0.19$ ,  $0.30 \pm 0.13$ , and  $1.39 \pm 0.12$   $\mu\text{g}/10^5\text{cells}$  for fibroblasts treated with medium only, 500 U/ml IFN- $\gamma$ , infected with Ad-GFP, or infected with Ad-IDO-GFP, respectively;  $P < 0.01$  relative to values of control group,  $n = 3$ ; Fig. 2.3C).

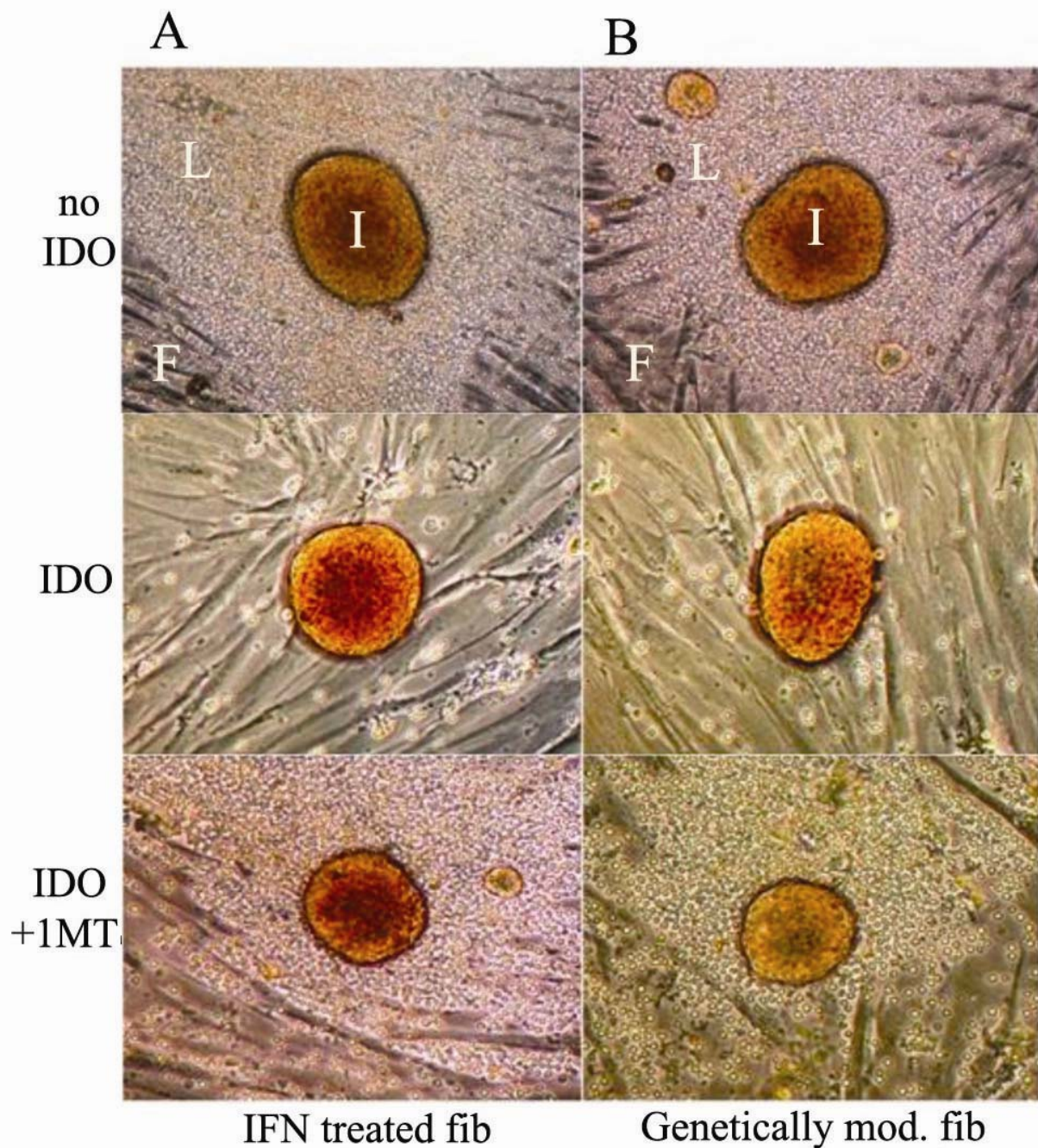


**Figure 2.3.** IDO expression in transduced fibroblasts at the levels of mRNA, protein, and kynurenine production. B6 mouse fibroblasts were treated with blank medium or IFN- $\gamma$  (500 U/ml); or infected with Ad-GFP vector or Ad-IDO-GFP vector at an MOI of 100. After 48 h of IFN- $\gamma$  treatment or 72 h post-infection, conditioned media were collected and cells were harvested. Part A shows Northern blot analysis for IDO mRNA. Total RNA of fibroblasts was extracted and expression of IDO mRNA was detected. Lanes from left to right are control (treated with blank medium), IFN- $\gamma$  treated, Ad-GFP infected, and Ad-IDO-GFP infected fibroblasts. 18S rRNA shows loading control. Part B shows western blot analysis of IDO protein. Upper arrow shows a 42 kDa band corresponding to the IDO protein. Expression of  $\beta$ -Actin protein was assessed as loading control. The order of the lanes is the same as those of Northern analysis. In part C, mean levels of kynurenine in conditioned media are shown. The kynurenine production rate is reported as  $\mu$ g per 10<sup>5</sup> cultured fibroblasts.\* Significantly different from the control value,  $P < 0.01$ ,  $n = 3$ .

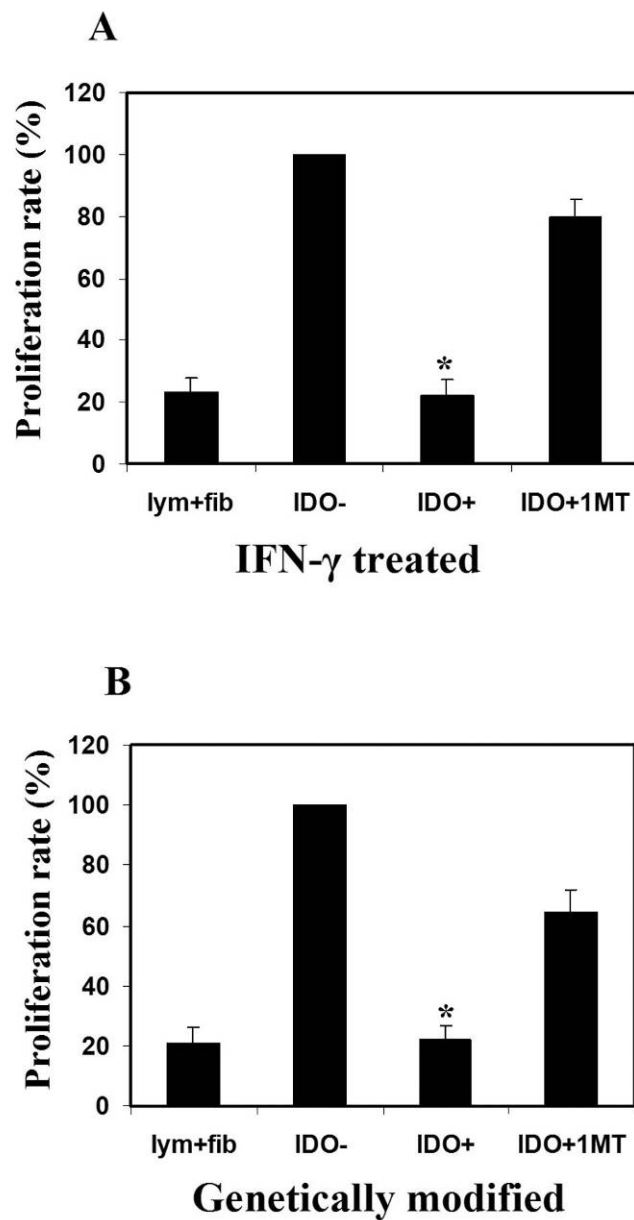


### **Effect of IDO expression on suppression of proliferation of lymphocytes co-cultured with allogeneic islets**

B6 mouse lymphocytes were stimulated *in vitro* with allogeneic BALB/c islets in the presence of B6 mouse fibroblasts treated with IFN- $\gamma$  or infected with Ad-IDO-GFP viral vector or control fibroblast. Photomicrographs were taken from this co-culture system after 72 h (Fig. 2.4). As shown in upper parts, lymphocytes (L) proliferated in response to stimulation by allogeneic islets (I) when co-cultured with untreated syngeneic fibroblasts (F). However, there was a remarkable reduction in the number of lymphocytes when co-cultured with allogeneic islets in the presence of IFN- $\gamma$  treated (Fig. 2.4A, middle parts) or genetically modified fibroblasts (Fig. 2.4B, middle part). The suppressive effect of IDO was eliminated in the presence of IDO inhibitor (Figs. 2.4A and B, lower parts). To confirm and quantify these findings, proliferation rates of lymphocytes were measured by [3H]-thymidine incorporation assay. The results showed a five-fold reduction in the proliferation rates of lymphocytes when co-cultured with allogeneic islets in the presence of syngeneic IDO-expressing fibroblasts compared to those of lymphocytes co-cultured with allogeneic islets and non-IDO-expressing fibroblasts in both IFN- $\gamma$  treated ( $21.9\% \pm 5.3$  vs. 100%,  $P < 0.01$ ,  $n = 5$ ; Fig. 2.5A) and genetically modified ( $22.1\% \pm 4.9$  vs. 100%,  $P < 0.01$ ,  $n = 5$ ; Fig. 2.5B) preparations. The suppression of immune cell proliferation was specific to IDO expression, because this effect was reversible upon addition of 1MT (Fig. 2.5A and B). Control B6 mouse lymphocytes co-cultured with B6 mouse fibroblasts showed no significant proliferation (the first bars in Fig. 2.5A and B).



**Figure 2.4.** Photomicrographs of co-cultured mouse lymphocytes with allogeneic islets and syngeneic IDO-expressing or control fibroblasts. B6 mouse fibroblasts (F) were treated with blank medium or 500 U/ml IFN- $\gamma$  or infected with Ad-IDO-GFP at an MOI of 100. After 48 h of IFN- $\gamma$  treatment or 72 h post-infection, fibroblasts were washed and BALB/c mouse islets (I) and B6 mouse lymphocytes (L) were added to the culture. Another experimental group received a competitive IDO inhibitor, 1-methyl-DL-tryptophan (1 MT) at the final concentration of 800  $\mu$ M. Photomicrographs were taken after 72 h of co-culture. Upper parts show lymphocytes (L) and islets (I) co-cultured with untreated control fibroblasts. Middle parts show the same preparation with IFN- $\gamma$  treated (left) and Ad-IDO-GFP infected (right) fibroblast. In the lower parts, 1 MT has been added to the co-culture. No IDO, fibroblasts cultured without IFN- $\gamma$  or virus; IDO, fibroblasts treated with IFN- $\gamma$  or infected with Ad-IDO-GFP; IDO +1 MT, fibroblasts treated with IFN- $\gamma$  or virus plus 1 MT. Magnification was at 100 $\times$ .



**Figure 2.5.** Proliferation rates of lymphocytes stimulated with allogeneic islets with or without IDO induction. B6 mouse fibroblasts were treated with 500 U/ml IFN- $\gamma$  or infected with Ad-IDO-GFP. These fibroblasts were then washed with PBS, irradiated and co-cultured with B6 mouse lymphocytes and irradiated BALB/c islets for 72 h. To one preparation in each experimental group a competitive IDO inhibitor, 1-methyl-DL-tryptophan (1 MT) was also added at the final concentration of 800  $\mu$ M. Control co-cultures of B6 mouse fibroblasts with B6 lymphocytes were also set up. Lymphocytes were then subjected to [3H]-thymidine incorporation assay and incorporation of [3H]-thymidine into their cellular DNA was evaluated after 16 h. Parts A and B show cells co-cultures with IFN- $\gamma$  treated or virally transduced fibroblasts, respectively. The bars show lymphocyte proliferation rates with the following order from left to right: co-culture of lymphocytes and syngeneic untreated fibroblasts, co-culture of lymphocytes with allogeneic islets and untreated syngeneic fibroblasts, co-culture of lymphocytes with allogeneic islets and IDO-expressing syngeneic fibroblasts, and co-culture of lymphocytes with allogeneic islets and IDO-expressing syngeneic fibroblasts plus 1 MT. The proliferation rates are standardized according to the counts per minute for lymphocytes co-cultured with untreated fibroblasts. Data are expressed as mean  $\pm$  SD. \* Significantly different from the control value,  $P < 0.01$ ,  $n = 5$ .

## 2.4 Discussion

The main finding of the present study is that bystander IDO-expressing syngeneic fibroblasts have the ability to suppress the allogenic lymphocytes proliferation which normally occurs in response to allogenic pancreatic islets. This study tested a novel approach to protect allogeneic islets through which local induction of IDO generates a tryptophan-deficient microenvironment in which immune cells are unable to survive. As a part of a series of experiments, here, we used two recombinant adenoviral vectors bearing either GFP as a reporter gene or IDO plus GFP genes and demonstrated that fibroblasts can successfully be infected and the resultant IDO is catalytically active in degradation of tryptophan. We also showed that IFN- $\gamma$  induces the expression of IDO mRNA and protein and that this induction was consistent with the functional enzyme activity by measuring the kynurenine in conditioned medium of treated and untreated cells. In a co-culture system, these IDO-expressing fibroblasts suppressed lymphocyte proliferation in response to allogeneic stimulation. This suppression is completely restored by the addition of an IDO inhibitor 1MT, indicating that suppression was due to IDO.

Previous studies have shown that IDO expression in different cell types such as dendritic cells (DCs) (6, 9, 30)(6), HELA cells (31), and dermal fibroblasts (12, 14, 15) can inhibit immune cell proliferation. IDO catalyzes tryptophan to N-formylkynurenine, which is then converted to kynurenine (3, 32), and because tryptophan is required for protein synthesis, stimulated immune cells would not be able to proliferate in such a low tryptophan environment. Others have suggested that tryptophan deprivation causes activated T cell apoptosis induced by expression of FasL (33). It is also reported that a tryptophan metabolite, 3-hydroxyanthranilic acid induces THP-1 and U937 cell apoptosis (34). In another study,

stimulation of CD8<sup>+</sup> DCs with IFN- $\gamma$ , which is known to promote IDO production, enhanced their tolerogenic activity toward CD4<sup>+</sup> T-cells (35). These, as well as similar findings obtained with IDO recombinant adenovirus transduced dendritic cells (6, 30) support the hypothesis that IDO expression by different cell types helps to maintain peripheral tolerance through regulating T cell response. Collectively, these results as well as our findings in the present study indicate that bystander immune cell damage induced by IDO maybe a key contributor for IDO-mediating immune tolerance and can work as an immunosuppressive factor.

Another remarkable aspect of IDO function is its selective suppressive effect. Although it has been demonstrated that IDO-mediated activated immune cell damage occurs via tryptophan depletion or accumulation of toxic metabolites, the degree of cell sensitivity to IDO seems to vary from one cell type to another. Our group has already shown that while high proliferating immune cells such as T-cells and Jurka T-cells are very sensitive to tryptophan-deficient environment, primary skin cells such as dermal fibroblasts, keratinocytes, and endothelial cells seem to be more resistant to low tryptophan (12). We have found that IDO-induced low tryptophan and high kynurenine culture environment does not have any negative impact on islet survival and function (please see Chapter 2). These findings suggest that local expression of IDO is likely to function selectively as a suppressive factor for infiltrated immune cells while does not put other cell types at risk.

There are two other reports that showed IDO can be used to improve islet graft survival in NOD mouse model. However they tried to infect the islets directly with an IDO adenovector (18) or a vector containing a combination of genes including IDO (19). We employed a different approach in the present study and used bystander syngeneic fibroblasts instead of

islets as IDO-expressing cells. We believe this approach would be beneficial in terms of islets survival and function because it has been shown that despite their advantages, adenoviral transduction and gene transfer may interfere with beta-cell function and/or induce cell death (22). Barbu et al. (23) have shown that even Bcl-2 overexpression in islet cells did not prevent adenoviral-induced toxicity, suggesting that the specific death pathway involved in adenoviral toxicity in beta-cells may bypass the mitochondrial permeability transition event. Another important limitation of the adenoviral-mediated gene transfer in beta cell research is the reduced ability to reach and transduce the inner cellular mass of the intact pancreatic islet (21). As such, fibroblasts are excellent candidates to be used as bystander IDO-expressing cells since these cells are common and regular cell type in connective tissue; easily induced to express IDO; and are resistant to IDO induced low tryptophan environment. Furthermore, availability of syngeneic or autologous fibroblasts eliminates the risk of any immune response against these cells.

In conclusion, the findings of this study show that local expression of IDO by syngeneic bystander fibroblasts can suppress immune cell proliferation when stimulated with allogeneic pancreatic islets *in vitro*. It remains to be determined whether our approach will result in significant prolongation of islet allograft survival in experimental animal models of transplantation. Using this approach, one can speculate that it is likely to develop a non-rejectable composite islet graft and provide a new therapeutic option for diabetic patients without compromising the functional efficacy of their immune system.

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## CHAPTER 3

### **Mouse Pancreatic Islets are Resistant to Indoleamine 2,3 Dioxygenase-Induced General Control Nonderepressible-2 Kinase Stress Pathway and Maintain Normal Viability and Function<sup>2</sup>**

#### **3.1 Introduction**

Indoleamine 2,3-dioxygenase (EC 1.13.11.52) (IDO) is a cytosolic monomeric hemoprotein that catalyzes tryptophan, the least available essential amino acid in the human body, to N-formylkynurenine, which in turn is rapidly degraded to yield kynurenine (1). IDO has been proposed to have profound immunoregulatory activity (2). IDO-dependent T-cell suppression by dendritic cells suggests that biochemical changes due to tryptophan catabolism have significant effects on T-cell proliferation and function (3). The regulatory effect of IDO on T cells is probably due to providing a tryptophan-deficient microenvironment and/or accumulation of toxic metabolites of tryptophan. The stress-responsive kinase general control nonderepressible 2 (GCN2) has been identified as a signaling molecule that enables T-cells to sense and respond to stress conditions created by IDO (4, 5). The C/EBP homologous protein (CHOP) gene is a downstream target gene in GCN2 pathway and is considered as a well-accepted marker for GCN2 activation (6).

It has been suggested that, due to its immunoregulatory effects, IDO may actively participate in down-regulating allogeneic immune responses in transplantation (7). Our research group has provided compelling evidence in support of the fact that the expression of

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<sup>2</sup> A version of this chapter has been published. Jalili RB, Forouzandeh F, Moeenrezakhanlou A, Rayat GR, Rajotte RV, Uludag H, Ghahary A. Mouse pancreatic islets are resistant to indoleamine 2,3 dioxygenase-induced general control nonderepressible-2 kinase stress pathway and maintain normal viability and function. *Am J Pathol.* 174:196-205, 2009 (<http://ajp.amjpathol.org/cgi/content/abstract/174/1/196>)

IDO in bystander fibroblasts through IDO genetic modification or interferon- treatment suppresses immune cell proliferation (8-13). In addition, we also have shown that, by an unknown mechanism, only immune but not primary skin cells are sensitive to the IDO suppressive effect (8, 14). In our recent study, we showed that bystander IDO-expressing syngeneic fibroblasts have the ability to suppress proliferation of lymphocytes stimulated by allogeneic mouse islets *in vitro* (15). This promising finding sets the stage for developing a nonrejectable composite graft consisting of islets and IDO-expressing fibroblasts embedded within a collagen scaffold. However, that study didn't elucidate whether IDO by itself has any deleterious effect on viability and functionality of islets. Here, we therefore asked whether a) exposing mouse islets to an IDO-induced low tryptophan microenvironment compromises their viability and function, b) the GCN2 pathway is activated in islets exposed to IDO-expressing cells, and c) a three-dimensional fibroblast populated collagen scaffold is a favorable matrix for constructing a composite islet graft.

## **3.2 Materials and Methods**

### **Mouse Islet Isolation and Culture**

Islets were obtained from 6 to 8-week-old male BALB/c or C57BL/6 (B6) mice (The Jackson Laboratories, Bar Harbor, ME) as previously described (15). Briefly, mice were anesthetized and pancreases were distended through the pancreatic duct with 2.5 ml of Hanks' balanced salt solution (Life Technologies, Gaithersburg, MD) containing 2.0 mg/ml of collagenase (Type V; Sigma Chemical Co., St Louis, MO). The distended pancreases were then removed and incubated at 37°C for 15 minutes. Then dissociated islets were purified by discontinuous centrifugation on Ficoll (Sigma) gradients. After centrifugation, islets were

handpicked and cultured in HAM's F10 medium (Sigma) supplemented with 12 mmol/L HEPES, 2 mmol/L L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in 95% air, 5% CO<sub>2</sub> at 37°C. Care and maintenance of all animals were in accordance with the principles of laboratory animal care and the guidelines of institutional Animal Policy and Welfare Committee.

### **Cell Co-cultures**

Cell co-cultures were set up using a two-chamber cell culture system (6-well plates, Corning incorporated, Corning, NY; cell culture inserts, Millicell, Millipore Corporation, MA) in which IDO-expressing fibroblasts were grown in the upper chambers while either lymphocytes, Jurkat cells, islets, or control fibroblasts were cultured in the lower chambers. To induce IDO expression, B6 mouse fibroblasts were infected with a recombinant IDO adenoviral vector carrying human IDO cDNA for 72 hours at a multiplicity of infection of 100, as previously described (15). Control fibroblasts were infected with a mock vector. Lymphocytes were isolated from peripheral lymph nodes of B6 mice by grinding lymph node tissues between the rough edges of glass slides and were stimulated with concanavalin A (2 µg/ml, Sigma) at the start of the culture. Specificity of the IDO effect was determined by addition of 1-methyl-tryptophan, an IDO inhibitor (Aldrich Chemical Co., Milwaukee, WI), to co-cultures at the final concentration of 800 µmol/L.

### **Development of Islet-Fibroblast Composite in Collagen Gel Matrix**

Mouse dermal fibroblasts were explanted from skin of B6 mice and transduced with a recombinant adenoviral vector carrying human IDO cDNA as described previously (15). Control fibroblasts were infected with a mock vector. Fibroblast-populated collagen gel matrices were prepared as described by Sarkhosh et al (12) using IDO-expressing or control

fibroblasts. Mouse islets were added to fibroblast-populated collagen gel before solidification in 24 well plates. The composites were maintained in 95% air, 5% CO<sub>2</sub> at 37°C for up to 14 days.

### **CHOP, IDO, and Insulin Reverse Transcriptase-PCR**

Total RNA was isolated using a RNeasy kit (Qiagen, Maryland). cDNA was synthesized using the ThermoScript reverse transcriptase (RT)-PCR System (Invitrogen, Carlsbad, CA). The primers used were as follows: CHOP: sense 5'-CATACACCACCACACCTGAAAG-3', antisense 5'-CCGTTTCCTAGTTCTTCCTTGC-3'; IDO: sense 5'-GGCACACGCTATGGAAACT-3', antisense 5'-CGGACATCTCCATGACCTTT-3'; mouse insulin 1: sense 5'-CCTGTTGGTGCACTTCCTAC-3', antisense 5'-TGCAGTAGTTCTCCAGCTGG-3'; glyceraldehyde-3-phosphate dehydrogenase: sense 5'-TGGCACAGTCAAGGCTGAGA-3' antisense 5'-CTTCTGAGTGGCAGTGATGG-3'. Amplified PCR products were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

### **GCN2 and CHOP Immunoblotting**

Cells were harvested and lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 10 mmol/L EDTA; 5 mmol/L EGTA; 0.5% NP40; 1% Triton X-100, and protease inhibitor cocktail, Sigma). Equal amounts of total protein from cell lysates (30 µg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). The blots were probed with the following antibodies: anti phospho-GCN2 (Thr898, 1:1000 dilution, Cell Signaling Technology INC., Beverly, MA), anti GCN2 (1:1000 dilution, Cell Signaling), and anti-GADD153/CHOP-10 produced in rabbit (1:250 dilution, Sigma). Horseradish peroxidase conjugated goat anti-rabbit IgG served as a

secondary antibody for the enhanced chemiluminescence detection system (Amersham Biosciences, UK).

### **IDO Immunoblotting and Kynurenine Measurement**

Fibroblasts were harvested and lysed 72 hours postinfection. Equal amounts of cell lysate were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane as described above. The blots were then probed with polyclonal anti IDO antibody raised in rabbit by Washington Biotechnology Inc. (Baltimore, MD) at a final dilution of 1:5000. Horseradish peroxidase conjugated goat anti-rabbit IgG served as a secondary antibody for the enhanced chemiluminescence detection system (Amersham). The level of kynurenine was measured as previously described (15). Briefly, proteins in conditioned medium were precipitated by trichloroacetic acid. After centrifugation, 0.5 ml of supernatant was incubated with equal volume of Ehrlich's reagent for 10 minutes at room temperature. Absorption of resultant solution was measured at 490 nm by spectrophotometer.

### **Methyl Thiazolyl Tetrazolium Proliferation Assay**

A colorimetric methyl thiazolyl tetrazolium (MTT) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used to evaluate the effects of IDO on cell proliferation. At the indicated time points, MTT (Sigma) solution (5 mg/ml) was added to cell cultures and incubated at 37°C for 5 hours. The formazin crystals were solubilized in 500 µl of dimethyl sulfoxide at the end of incubation, and the optical density of the solutions was measured at 570 nm.

## **Insulin Immunostaining**

Islets were recovered and fixed in Bouin's solution for 2 hours. Islets were then washed three times with 70% ethanol and embedded in paraffin. Five micron sections of these samples were stained with guinea pig anti-insulin antibody (1:1000 dilution; Dako Laboratories, Mississauga, ON, Canada) for 30 minutes followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA). The avidin-biotin complex/horseradish peroxidase (Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochloride (BioGenex, San Ramon, CA) was used to produce a brown positive reaction. All sections were counterstained with Harris' hematoxylin and eosin (H&E).

## **$\beta$ Cell Apoptosis**

Cleaved caspase-3 was used as a marker for  $\beta$ -cell apoptosis. To estimate  $\beta$  cell apoptosis rates, islets were stained for cleaved caspase-3 together with insulin.  $\beta$  cell apoptosis rates were then estimated by calculating the frequency of the cells positively stained for both insulin and cleaved caspase-3. Insulin/cleaved caspase-3 dual immunofluorescence staining was accomplished as follows: islets were retrieved and fixed as described above. Five-micron sections were incubated overnight at 4°C with guinea pig anti insulin antibody (1:500 dilution, Dako) and rabbit anti-cleaved caspase-3 antibody (1:100 dilution, cell signaling, Beverly, MA). After three washing steps with PBS-Tween 20 for 5 minutes each, samples were incubated with fluorescein-conjugated donkey anti-rabbit antibody (1:200 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) and rhodamine-conjugated anti-guinea pig antibody (1:200 dilution, Abcam, Cambridge, MA) for 45 minutes in the dark. Finally, after washing with PBS-Tween 20 three times for 5 minutes



each, samples were mounted in Vectashield H-1200 (Vector Laboratories) containing 4,6-diamidino-2-phenylindole for nuclei staining. A Zeiss Axioplan 2 microscope and Northern Eclipse image analysis software were used to obtain the images.

### **Static Incubation Assay and Islet Insulin Content**

Islet insulin secretory responsiveness was assessed using a static incubation assay as described by Korbitt et al (16). In brief, mouse islets were washed twice with Ham's F10 medium and samples were taken for measurement of total cellular insulin content. Islets were cultured in 24-well plates and incubated in 1.5 ml of HAM's F10 medium supplemented with 0.5% bovine serum albumin and either 2.8 or 20.0 mmol/L glucose for 120 minutes. At the end of the incubation, supernatants were collected for measurement of insulin release using radioimmunoassay. Insulin secretion was calculated by dividing the insulin released into the supernatant by the cellular insulin content of the islets (percentage of content). Stimulation indices were calculated by dividing the percentage of insulin released at 20.0 mmol/L glucose by the percentage released at 2.8 mmol/L glucose.

Determination of islet total insulin and DNA content was performed as described by Korbitt et al (16). In brief, samples were sonicated in 2 mmol/L acetic acid containing 0.25% bovine serum albumin and centrifuged (800 x g, 15 minutes). Insulin levels in the supernatants were measured in duplicate samples by radioimmunoassay (Diagnostic Products, Los Angeles, CA). The islet DNA content was quantified using PicoGreen kit (Molecular Probes, Eugene, OR) according to the manufacturer's instruction.

### **Transplantation of Islet-Fibroblast Composite Grafts**

Recipient B6 mice were rendered diabetic by a single intraperitoneal injection of 200 mg/kg streptozotocin (Sigma), and diabetes was defined as a minimum of two consecutive

blood glucose measurements  $\geq 20$  mmol/L. Syngeneic islet plus IDO-expressing or control fibroblast composite grafts (approximately 500 islets) were transplanted under the left kidney capsule of isoflurane-anesthetized diabetic mice. After transplantation, blood from the tail vein of each recipient was collected two times a week between 7:00 and 9:00 AM to determine the normalization of blood glucose levels. Blood glucose levels were measured using a One Touch Ultra glucose meter (Lifescan, Milpitas, CA), and grafts were deemed functioning when blood glucose levels decreased to  $<10.0$  mmol/L. Nephrectomy of the graft-bearing kidney was performed on recipients at the endpoint of the study ( $>100$  days post-transplant) to confirm that hyperglycemia ensued, indicating that normal blood glucose was graft dependent. All animals were cared for according to the guidelines of the Institutional Animal Policy and Welfare Committee.

### **Intraperitoneal Glucose Tolerance Test**

An intraperitoneal glucose tolerance test (IPGTT) was performed in mice transplanted with composite grafts 6 weeks after transplantation. After a 16-hour overnight fast, glucose (2 mg/g body weight) was injected i.p. into nonanesthetized mice. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120 minutes. Area under the curve was determined using SigmaPlot software (Systat Software Inc., San Jose, CA).

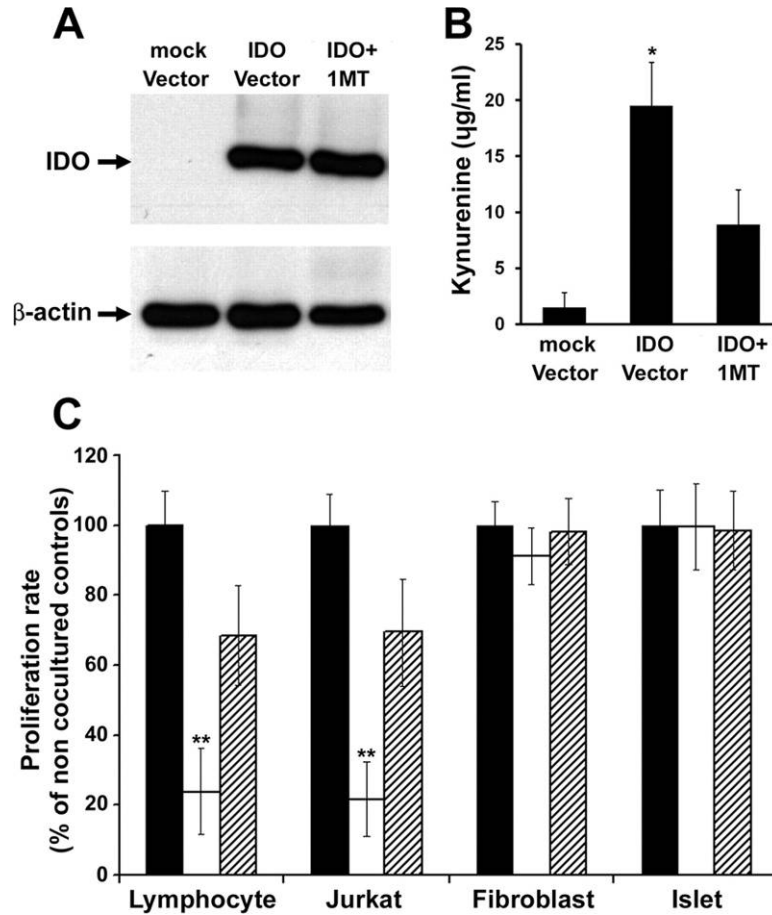
### **Statistical Analysis**

All data are reported as mean  $\pm$  SD of three or more independent observations. Statistical significance was calculated using a two-tailed unpaired Students' t-test or a one-way analysis of variance with post hoc test in case of multiple comparisons. P values less than 0.05 were considered to be significant.

### 3.3 Results

#### Selective Suppressive Effect of IDO on Immune Versus Islet Cells

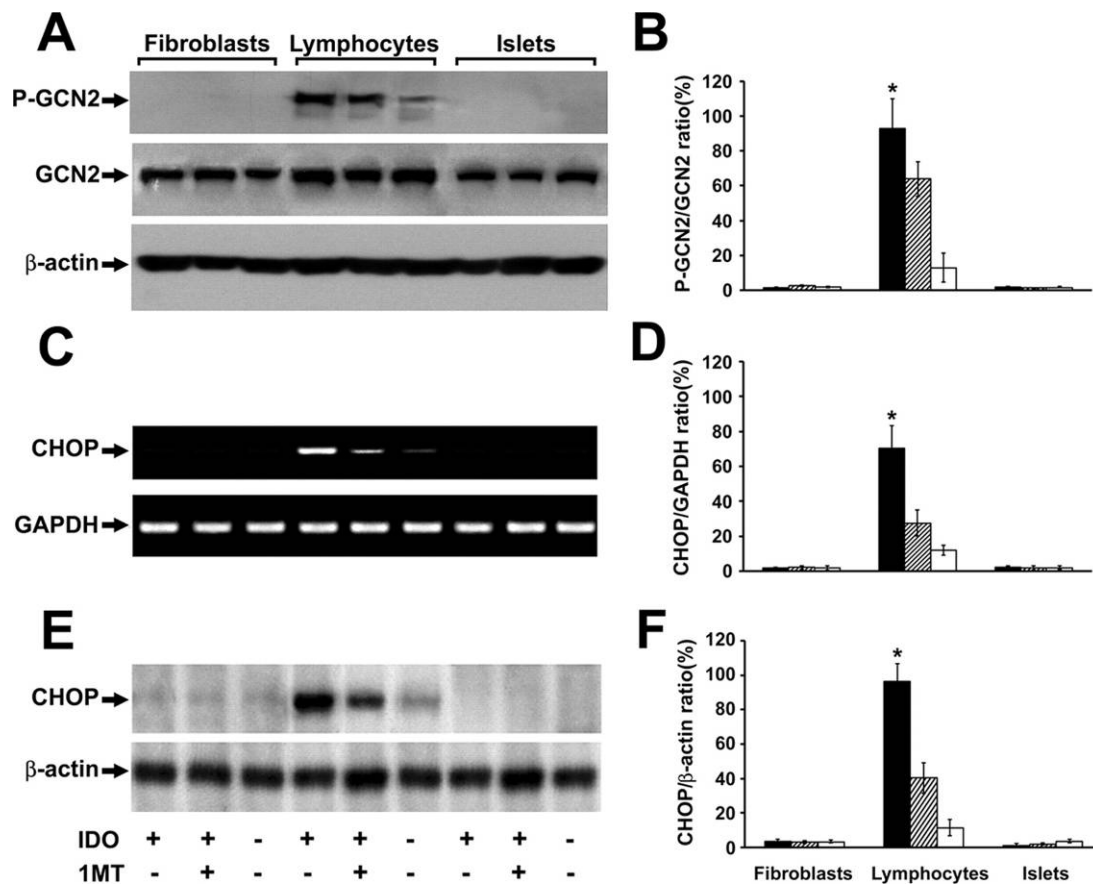
To confirm the differential suppressive effect of IDO on immune versus non-immune cells, we induced IDO expression in B6 mouse dermal fibroblasts using a recombinant adenoviral vector expressing IDO. Expression of IDO protein in infected cells was shown using western blot analysis (Figure 3.1A). High levels of kynurenine - a tryptophan metabolite - in conditioned media of IDO vector infected cells further confirmed enzymatic activity of IDO (Figure 3.1B). IDO-expressing fibroblasts were then co-cultured with stimulated mouse lymphocytes, CD4<sup>+</sup> Jurkat cells, mouse fibroblasts, or islets using a two-chamber co-culture system. The results of a MTT assay on co-cultured cells after 72 hours showed significant reduction in cell proliferation rates in lymphocytes ( $23.9\% \pm 12.4$ ) and Jurkat cells ( $21.8\% \pm 10.7$ ) but not in fibroblasts and islets (Figure 3.1C). Proliferation of immune cells did not decrease when co-cultured with control fibroblasts. Furthermore, addition of 1-methyl-tryptophan, a specific IDO inhibitor, resulted in partial recovery of immune cells proliferation ( $68.5\% \pm 14.3$  in lymphocytes and  $69.4 \pm 15.3$  in Jurkat cells, Figure 3.1C).



**Figure 3.1.** Selective suppressive effect of IDO on immune versus non immune cells. Mouse fibroblasts were transduced to express IDO using an adenoviral vector. A: IDO protein expression in mock and IDO vector infected cells. The level of kynurenine (the product of IDO mediated tryptophan degradation) was measured in the conditioned media (B). C: Stimulated mouse lymphocytes, Jurkat cells, mouse fibroblasts, and islets were co-cultured in two-chamber culture plates with IDO-expressing (open bars) or control fibroblasts (solid bars) for 72 hours. A competitive IDO inhibitor, 1-methyl-tryptophan was added to one set of IDO-expressing co-cultures (hatched bars). Cell proliferation rates were measured after 72 hours post-coculture using MTT assay. \* denotes significant increase in kynurenine level in IDO vector infected cell conditioned medium compared to the control group (n = 3, P < 0.001); \*\* denotes significant difference in cell proliferation rate in comparison to the control group (n = 5, P < 0.001).

### **GCN2 Kinase Pathway Activation in Cells Exposed to IDO-Expressing Fibroblasts**

The activation of the GCN2 kinase pathway is suggested as the downstream mechanism for the suppressive effect of IDO. We therefore asked whether this stress-response mechanism is selectively activated in different cell strains when co-cultured with IDO-expressing cells. To address this question, we examined phosphorylation of GCN2 and induction of intracellular CHOP in stimulated lymphocytes, fibroblasts, and islets co-cultured with IDO-expressing fibroblasts. The results shown in the Figure 3.2 indicate a differential pattern of GCN2 pathway activation in immune versus islet cells in response to IDO. As shown in the Figure 3.2A, co-culture with IDO-expressing cells promotes phosphorylation of GCN2 in mouse lymphocytes but not in islets and fibroblasts. Similarly, on exposure to IDO, CHOP was induced only in mouse lymphocytes at mRNA (Figure 3.2C) and protein (Figure 3.2E) levels. The quantitative analyses shown in Figure 3.2, B, D, and F indicated a significant and selective increase (more than sevenfold) in GCN2 phosphorylation and CHOP message and protein levels in lymphocytes co-cultured with IDO-expressing cells that is partially reversed on IDO inhibition with 1-methyl-tryptophan.

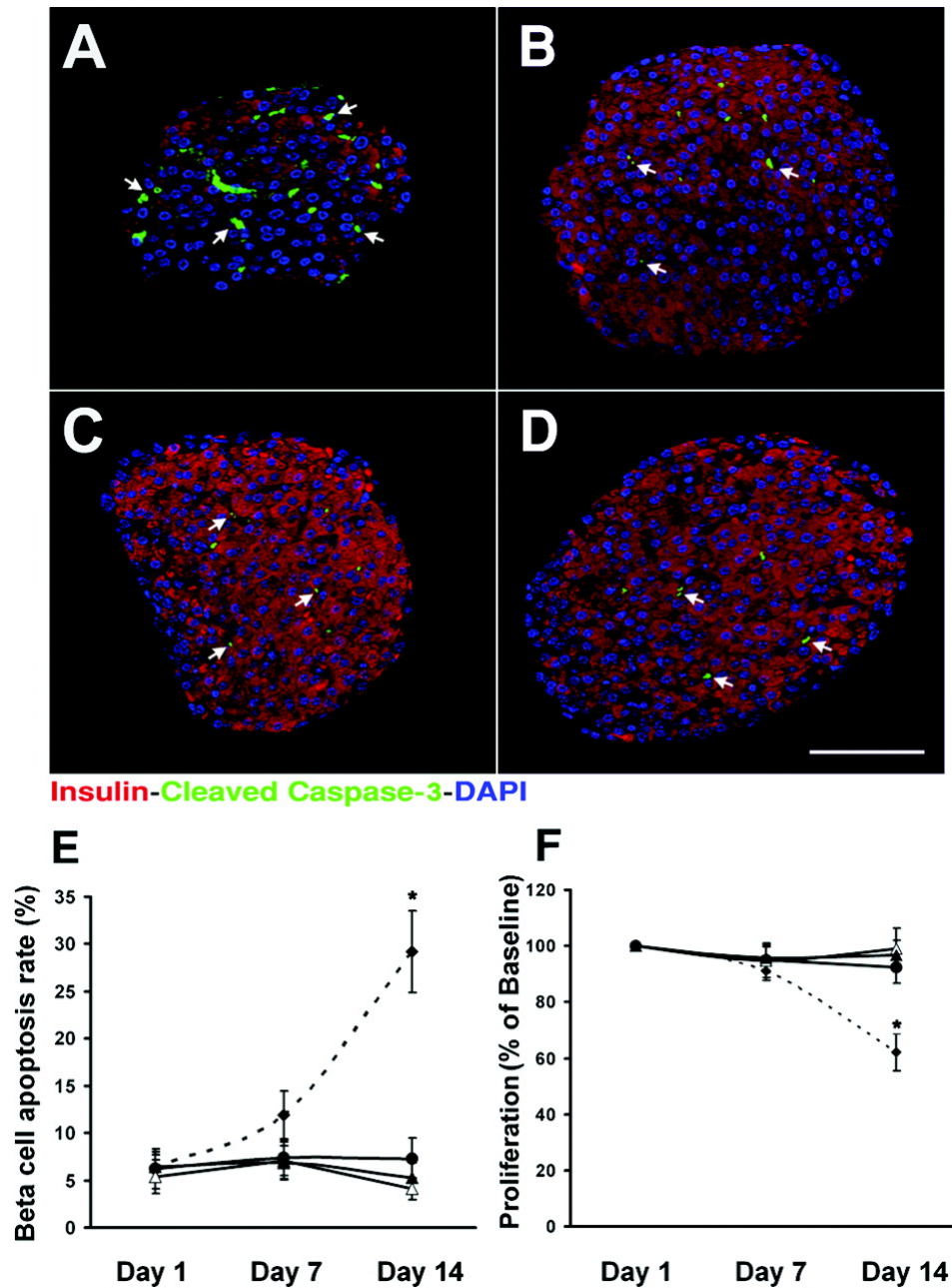


**Figure 3.2.** Selective activation of GCN2 and CHOP expression in immune cells as compared to islets and fibroblasts. Stimulated mouse lymphocytes, fibroblasts, or islets were co-cultured with IDO-expressing or control fibroblasts for 48 hours. The phosphorylation of GCN2 and induction of CHOP was then measured. A competitive IDO inhibitor, 1-methyl-tryptophan was added to one set of IDO-expressing co-cultures. A: phopho-GCN2 (upper row) and total GCN2 (middle row) western blot. C: CHOP RT-PCR result. E: result of western blot analysis for CHOP. Upper arrow shows a 29 kDa band corresponding to CHOP. B, D and F: the mean ratio of densities of phospho-GCN2, CHOP message, and protein bands to those of total GCN2, glyceraldehyde-3-phosphate dehydrogenase-1, and β-actin bands, respectively, in cells co-cultured with either IDO expressing (solid bars), IDO expressing plus 1-methyl-tryptophan (hatched bars), or control (open bars) fibroblasts. \* denotes significant difference in phospho-GCN2 and CHOP level between the IDO exposed and the control lymphocytes ( $n = 3$ ,  $P < 0.001$ ). P-GCN2: phosphorylated GCN2.

## **Viability of Islets Embedded within IDO-Expressing Fibroblast Populated Collagen Matrix**

Embedding islets within an extracellular matrix generally improves islet survival and function. However, the impact of local IDO expression on enhanced viability of islets within three-dimensional matrix has not been elucidated. To investigate the effect of IDO on survival of islets within a collagen gel scaffold, we prepared three-dimensional composite co-cultures embedded with mouse islets plus either IDO-expressing or control fibroblasts. A total of 100 islets and 100,000 fibroblasts were included in each experimental group. These composites were then incubated *in vitro* for up to 2 weeks. On days 1, 7, and 14 post co-culture, the collagen matrices were digested and islets were retrieved and subjected to apoptosis and MTT assays. In parallel, one set of mouse islets was cultivated in a regular two-dimensional setting as a control.

Islet double-immunofluorescence staining of cleaved caspase-3 and insulin was used to estimate  $\beta$ -cell apoptosis rates. As shown in the Figure 3.3A–E,  $\beta$  cell apoptosis rates were significantly lower in matrix embedded islets and furthermore, being in close vicinity of IDO-expressing fibroblasts for 2 weeks did not increase  $\beta$ -cell apoptosis rate ( $5.3\% \pm 1.5$  vs.  $4.1\% \pm 1.2$ ,  $P > 0.05$ ). The MTT assay result indicated that, in contrast to two-dimensional culture, the islet cell proliferation rate was maintained near baseline level when embedded within the collagen matrix, regardless of being either alone, or co-cultured with IDO-expressing or control fibroblasts. Additionally, MTT assays confirmed IDO did not significantly decrease the islet cell proliferation rate in composites containing IDO-expressing cells ( $96.8\% \pm 5.3$ ) compared with control composites ( $99.2\% \pm 7.1$ ;  $P > 0.05$ , Figure 3.3F).



**Figure 3.3.**  $\beta$  cell apoptosis and proliferation rates of islets embedded within fibroblast populated collagen gel. Mouse islets were embedded within IDO-expressing or control scaffolds or cultivated in regular petri dishes as described in the Materials and Methods. On days 1, 7, and 14 of co-culture, islets were harvested and subjected to insulin/cleaved caspase-3 dual immunofluorescence staining and MTT assay. The four upper panels show insulin (red)/cleaved caspase-3 (green) dual immunofluorescence staining in islets cultured in petri dishes (A), embedded within either acelluar gel (B), control fibroblast gel (C), or IDO fibroblast gel (D) for 14 days. (E and F) show  $\beta$  cell apoptosis and islets proliferation rates in islets embedded in IDO-expressing (solid triangles) or control fibroblast (open triangles) populated or acelluar (solid circles) collagen gel matrices and islets cultured in petri dishes using regular two-dimensional culture method (solid diamonds- dotted line) on days 1, 7, and 14 post co-culture. Islet proliferation rates are reported as the percentage of the optical densities of MTT assay at each time point adjusted to those of day 1. \* denotes significant difference in apoptosis and proliferation rates on day 14 compared to day 1 ( $n = 3$ ,  $P < 0.001$ ). White arrows show representative islet cells stained for both insulin and cleaved caspase-3. Scale bar=50  $\mu$ m.

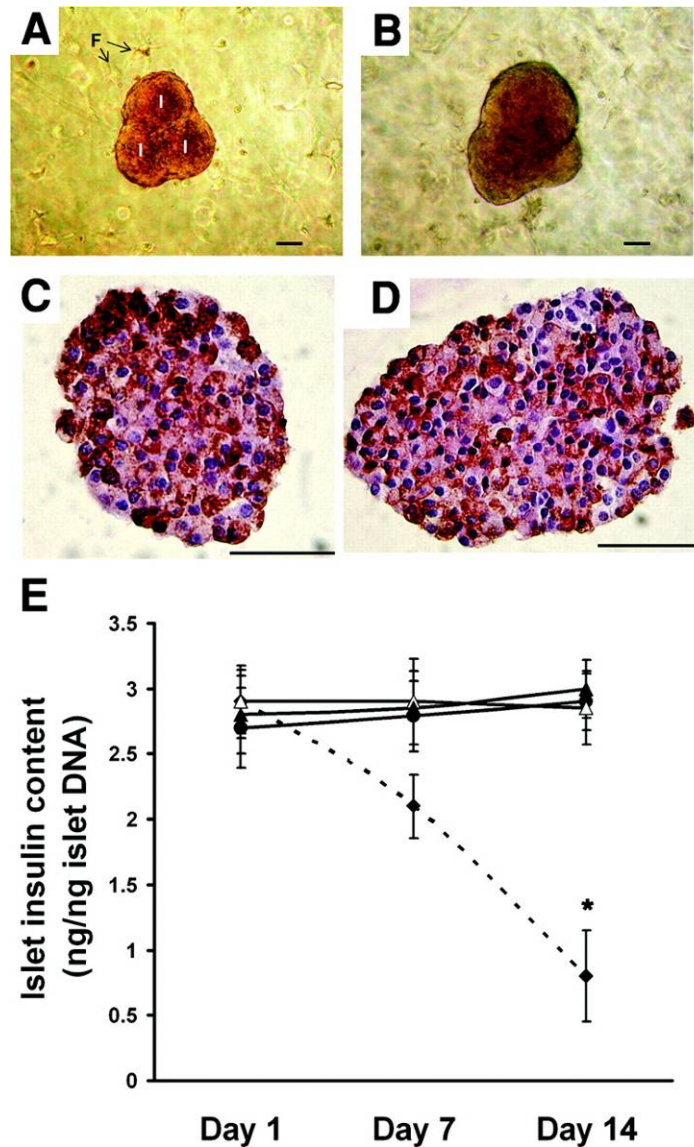


### **Morphology, Insulin Content and Functional Capacity of Islets Embedded within an IDO-Expressing Fibroblasts Populated Collagen Matrix**

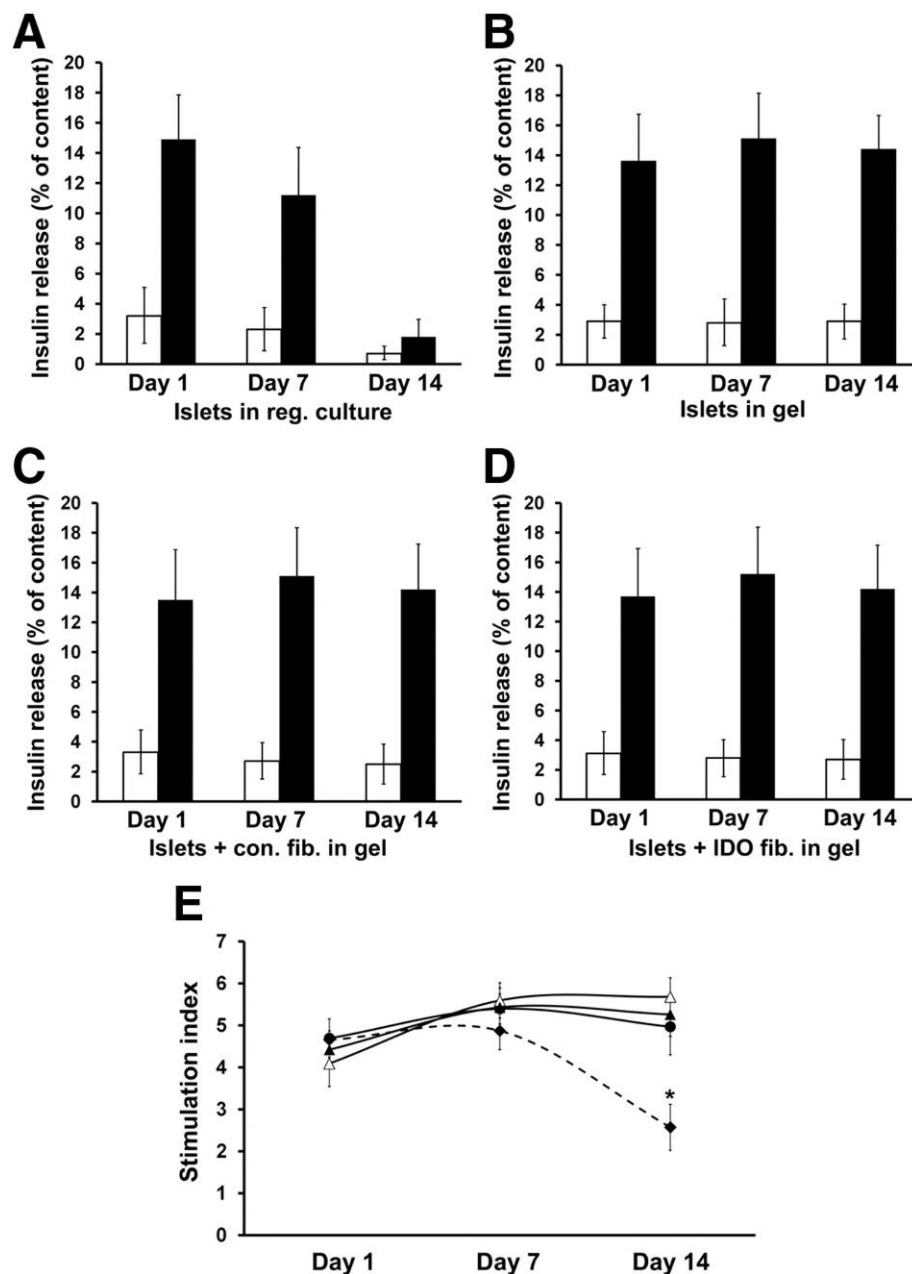
To gain perspective on the effect of IDO on islet functionality, the islets that were embedded within the three-dimensional composites were retrieved on days 1, 7, and 14 post co-culture and subjected to glucose-stimulated insulin secretion assay and insulin immunostaining. Total insulin content of islets was also measured. Figure 3.4, A–D shows photomicrographs of the composite islet-fibroblast-collagen matrix preparations, indicating normal spherical morphology of islets with smooth borders (Figure 3.4, A and B). Insulin immunostaining confirmed that the insulin content of islets exposed to IDO-expressing fibroblasts within a collagen scaffold for 2 weeks (Figure 3.4C) is comparable with islets co-cultured with control fibroblasts under similar experimental condition (Figure 3.4D). Furthermore, total insulin content of islets exposed to IDO-expressing fibroblasts for 2 weeks was  $3 \pm 0.22$  ng/ng islet DNA, which was not significantly different from insulin content of control islets ( $2.85 \pm 0.28$  ng/ng islet DNA, Figure 3.4E).

The insulin secretory capacity of islets was tested by comparing the percentages of cellular insulin released in low glucose (2.8 mmol/L) versus high glucose (20 mmol/L) media. The result showed a significant decrease in the insulin secretion ability of the islets cultured in the regular two-dimensional setting for 14 days (Figure 3.5A). However, glucose responsiveness and insulin secretory capacity remained at normal levels in the islets embedded within collagen matrix for 14 days, regardless of co-culture conditions used (Figure 3.5, B–D). The calculated islet stimulation index did not significantly change in the islets co-cultured with IDO-expressing fibroblasts for 14 days ( $5.26 \pm 0.52$ ) compared with the baseline ( $4.42 \pm 0.45$ ), whereas islet stimulation index significantly decreased in the islets

cultivated in a two-dimensional setting from  $4.66 \pm 0.50$  at the baseline to  $2.57 \pm 0.55$  after 2 weeks (Figure 3.5E ,  $n = 3$ ,  $P < 0.001$ ).



**Figure 3.4.** Morphology, insulin immunostaining, and total insulin content of islets embedded within fibroblast populated collagen scaffold. Mouse islets were embedded in IDO-expressing or control fibroblast populated collagen gel matrices for up to 14 days. Upper panels show photomicrographs of composite islet co-cultures in IDO fibroblast (A) and control fibroblast (B) populated collagen matrix. Islets co-cultured with IDO fibroblasts (C) or control fibroblasts (D) were harvested after 14 days and immunostained for intracellular insulin. Panel (E) shows total insulin content of islets embedded within IDO-expressing (solid triangles) or control fibroblast (open triangles) populated or acellular (solid circles) collagen gel matrices and islets cultured in petri dishes using regular two-dimensional culture method (solid diamonds-dotted line) on days 1, 7, and 14 post-coculture. \* denotes significant difference in total insulin content on day 14 compared to day 1 ( $n = 3$ ,  $P < 0.001$ ). I = islet, F = fibroblast. Scale bar = 50  $\mu\text{m}$ .



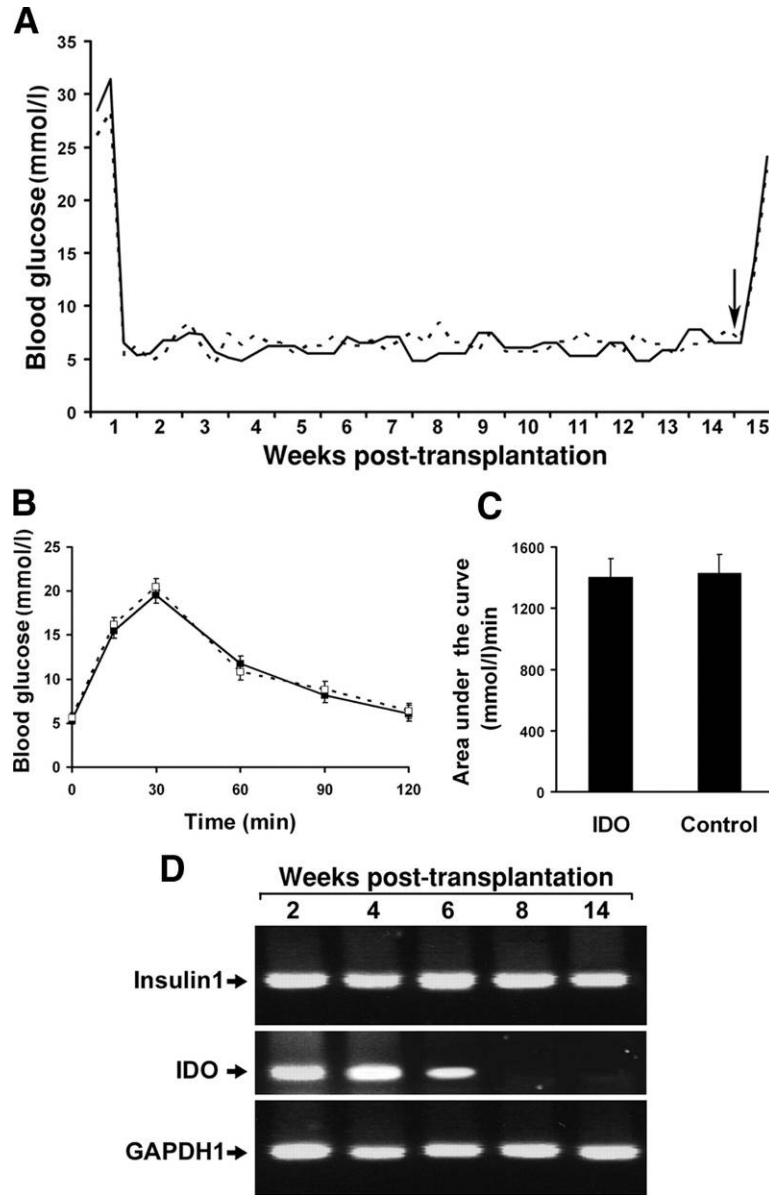
**Figure 3.5.** Capacity of glucose-mediated insulin secretion in islets embedded within fibroblast populated collagen matrix. Mouse islets were embedded within IDO-expressing or control scaffolds or in regular petri dishes as described in the Materials and Methods. On days 1, 7, and 14 of co-culture, islets were harvested and subjected to static incubation assay to test their glucose-stimulated insulin secretory capacity at low (2.8 mmol/L, open bars) versus high (20.0 mmol/L, solid bars) glucose concentrations. Insulin release rates were measured in islets cultured in petri dishes (A), embedded within either acellular gel (B), control fibroblast gel (C), or IDO fibroblast gel (D). The data are reported as the percentage of the released insulin to total insulin content of the islets. (E) Shows islet stimulation indices, which were calculated by dividing the percentage of insulin released at high glucose by the percentage released at low glucose concentrations. Solid diamonds-dotted line, islets cultured in regular petri dishes; solid circles, islets embedded within acellular gel; open triangles, islets embedded within control fibroblast gel; solid triangles, islets embedded within IDO fibroblast gel. \* denotes significant decrease in islet stimulation index on day 14 vs. day 1 ( $n = 3$ ,  $P < 0.001$ ).

### **Transplantation of a Syngeneic Islet-Fibroblast Composite Graft**

To confirm that islets embedded within the IDO-expressing fibroblast populated collagen scaffold are also viable and functional in a syngeneic transplantation model, composite grafts were prepared by embedding B6 mouse islets and IDO-expressing or control fibroblasts within the collagen matrix. These composite grafts were then transplanted beneath the renal capsule of chemically induced diabetic B6 mice. Blood glucose levels became normal in all graft recipient animals after 2 to 3 days post-transplantation and remained normal throughout the experiment (Figure 3.6A). On day 100 post-transplantation, removal of the graft bearing kidneys in graft recipient animals resulted in recurrence of hyperglycemia, which confirmed graft related euglycemia induction. To further investigate composite islet graft function, an IPGTT was performed in graft recipient mice on week 6 post-transplantation. Blood glucose concentrations during IPGTT were similar in IDO composite graft recipients and controls (Figure 3.6B). Comparing the area under the IPGTT curve showed similar graft function in IDO group compared to the control group ( $1401 \pm 120$  (mmol/L) min vs.  $1430 \pm 135$  (mmol/L) min, respectively;  $P > 0.05$ , Figure 3.6C).

To examine the length of adenovirus-mediated IDO transgene expression in the composite grafts, IDO mRNA level was measured in the grafts using RT-PCR at different time points post-transplantation. As shown in Figure 3.6D, IDO is strongly expressed in the IDO composite grafts for up to 6 weeks after transplantation. High levels of insulin 1 expression were also detected in the grafts throughout the experiment (Figure 3.6D), which further confirmed maintenance of functional islets in the composite grafts during and after the IDO transgene expression period. These findings collectively indicate long term survival

and normal functionality of islets exposed to IDO-expressing cells in a syngeneic islet transplantation model.



**Figure 3.6.** Syngeneic islet-fibroblast composite graft survival and intragraft IDO transgene expression *in vivo*. A: B6 mouse islets were embedded within IDO-expressing (solid line) or control fibroblast (dotted line) populated collagen matrices and transplanted to the kidney subcapsular spaces of chemically induced diabetic B6 mice (3 mice per group). Blood glucose levels of graft recipient animals were measured twice a week for 100 days. On day 100 post-transplantation (black arrow), graft bearing kidneys were removed, which resulted in recurrence of hyperglycemia (A). IPGTT was performed in graft recipients on week 6 post-transplantation. (B) and (C) show blood glucose concentrations during IPGTT and the area under the IPGTT curve, respectively. (D) Shows result of intragraft insulin 1 (upper row) and IDO RT-PCR (middle row) at the end of weeks 2, 4, 6, 8, and 14 post-transplantation.

### 3.4 Discussion

In this study, we report that the viability and functionality of islets exposed to IDO-expressing fibroblasts are not compromised *in vitro* and *in vivo*, using different experimental approaches. The significance of this finding is more appreciated when the potential application of local expression of IDO is considered as a strategy for protecting islet grafts from immune rejection. IDO is a potent immunomodulatory enzyme that plays critical roles in regulation of T cell-mediated immune responses and has profound effects on T-cell proliferation, differentiation, effector functions and viability (3). Considerable evidence now supports the importance of the immunoregulatory function of IDO, including studies of mammalian pregnancy (17-19), tumor resistance (20-23), chronic infections (24-26) and autoimmune diseases (27). Based on these facts, it has been suggested that cells expressing IDO might be used to protect transplanted tissues and cells without the use of immunosuppressive drugs. We have recently introduced a model of a local immunosuppressive barrier to protect immune responses against islets in which syngeneic bystander IDO-expressing fibroblasts suppress lymphocyte proliferation induced by allogeneic mouse islets (15). Our model is based on development of a three-dimensional composite graft consisting of islets and IDO-expressing fibroblasts embedded within a collagen matrix.

Although the selective suppressive effect of IDO on T cells versus skin cells has already been noted (8, 14), there was no evidence on the impact of low tryptophan microenvironment induced by IDO on other cell types including islets. We therefore, as shown in the Figures 3.1 and 3.3, tested the proliferation rates of mouse islets and fibroblasts when co-cultured with IDO-expressing cells. The results of these experiments confirmed that

exposure to IDO-expressing cells did not decrease islet cell proliferation or increase the  $\beta$  cell apoptosis rate *in vitro*.

The molecular mechanism(s) by which IDO suppresses T cells are still being investigated. It appears that some of the biological effects of IDO can be mediated via local depletion of tryptophan (28, 29), whereas others are mediated via immunomodulatory tryptophan metabolites (30, 31). As mentioned earlier, activation of the GCN2 kinase pathway, a nutrient deficiency stress-responsive mechanism, has been suggested as a potential mechanism responsible for the IDO-induced suppressive effect on T-cells (4, 5). Activation of the GCN2 kinase pathway can trigger cell-cycle arrest, differentiation, compensatory adaptation, or apoptosis, depending on the cell type and the initiating stress (32, 33). CHOP (also known as GADD153) is a DNA damage-inducible, nuclear leucine zipper protein involved in differentiation and apoptosis. CHOP is a downstream target gene in GCN2 pathway and a well-accepted marker for GCN2 activation (6). Its expression is induced in a variety of stress responses such as endoplasmic reticulum stress (34), redox stress (35), and nutrient deprivation (36, 37). Functional roles for CHOP have been well described in induction of apoptosis, and it has also been implicated in the pathogenesis of diabetes by promoting  $\beta$  cell destruction (38). This study for the first time showed that GCN2 activation and CHOP expression did not occur in mouse islets in response to IDO exposure whereas GCN2-mediated CHOP expression increased more than sevenfold in mouse lymphocytes under similar experimental condition. Thus, the selective unresponsiveness of mouse islets to IDO-induced GCN2 activation and CHOP expression suggests that islet cells are IDO resistant due to lack of GCN2 pathway responsiveness to IDO-induced low tryptophan environment.

Although the mechanism(s) underlying selective GCN2-CHOP pathway activation in immune versus islet cells needs to be further elucidated, at the present time, there are, at least, three potential explanations. First, activation of the GCN2 pathway is a nutrient deficiency stress response, and therefore actively dividing cells including highly proliferating T-cells, but not non-dividing islet cells, would be more sensitive to this environment. In fact, it has been shown that activated and proliferating T-cells, but not resting T cells, cultured in IDO-induced low tryptophan environment rapidly undergo apoptosis (39). Moreover, Mellor et al showed that suppression of T-cell proliferation in the presence of IDO-expressing cells occurred after the majority of T-cells entered the cell proliferation cycle (40). Second, there may be a compensatory mechanism by which islet cells suppress IDO mediated GCN2 activation. This suggestion is based on the fact that Pereira et al recently showed that the mouse protein IMPACT, which binds GCN1 and inhibits GCN2 activation, abolishes the expression of its downstream target genes ATF4 and CHOP (41). It should be emphasize that IMPACT is highly expressed in IDO-expressing tissues (e.g., placenta and testis) and also in pancreas (42). As such, the expression of IMPACT in pancreatic islet cells might function as a protective mechanism against IDO-induced GCN2 activation. Finally, a different and high affinity transport system for tryptophan may exist in IDO resistant cells such as islet cells. Recently, a novel amino acid transport activity with high affinity and unusual selectivity for tryptophan has been described in IDO-expressing human monocyte-derived macrophages, which is up-regulated during monocyte-derived macrophage differentiation but not in T-cells (43). This selective transport system, if it exists in other IDO resistant cell types, can enhance tryptophan uptake in these cells and therefore overcome nutrient deficiency stress response in a low tryptophan environment.



The collagen matrix in our composite grafts plays a dual role since it works both as a scaffold to keep islets and fibroblasts together and therefore maintains the integrity of the graft and also works as an extracellular matrix, which improves the survival and function of islets. Extracellular matrix is one of the most important constituents of the islet microenvironment. It is well documented that when interrelationship between islets and extracellular matrix is disrupted following harsh process of islet isolation and purification, the function and survival of isolated islets are significantly compromised (44, 45). It was also noted that islets cultivated in regular two-dimensional setting in petri dishes gradually disintegrate during culture in a time dependent manner (46). Therefore, it has been suggested that entrapment of islets in a three-dimensional collagen matrix would maintain satisfactory morphology, viability, and glucose-induced insulin secretory capability in islets (47). Our findings regarding improvement of cell survival and glucose stimulated insulin secretory capacity of the islets embedded within a collagen matrix, regardless of co-culture conditions, are in concordance with previous studies (48-50) and confirm that embedding islets in a scaffold consisting collagen will maintain integrity of islets and improve their viability and function.

Fibroblasts co-cultured with islets in our proposed composite model can also improve islet cell viability. The essential role of islet-derived fibroblasts in islet physiological competence was previously reported, and it was shown that some fibroblast produced factors can promote islet survival in culture (51). Miki et al recently showed that co-culture of the mouse, rat, and pig islets with islet-derived fibroblasts maintained islet viability, insulin secretion and glucose responsiveness (52). Co-cultured fibroblast would not cause any concern in terms of overproliferation since it was shown that fibroblasts in floating collagen

matrices have low levels of DNA synthesis and become quiescent (53-55). Thus, bystander fibroblasts will not have any negative impact on islet viability and function.

The findings shown in the Figure 3.6 further confirm the long term survival and functionality of syngeneic islets within the composite grafts in a diabetic animal model. The IDO transgene was strongly expressed in the composite grafts for up to 6 weeks post-transplantation, as expected following gene transfer using adenoviral vectors. The level of insulin mRNA was high in the IDO grafts throughout the experimental period. These data collectively demonstrate that none of key elements in our composite islet graft including IDO enzymatic activity, fibroblasts, and collagen matrix has a negative impact on long term viability and function of transplanted islets.

In conclusion, the findings of the present study suggest that mouse islets are selectively resistant to IDO-mediated activation of GCN2 kinase stress pathway and that coculture of mouse islets in a three-dimensional IDO-expressing fibroblast populated collagen matrix does not have any deleterious effect on viability and insulin secretory capacity of islets *in vitro* and *in vivo*. These findings together with the already proven immunosuppressive effect of IDO set the stage for developing a nonrejectable islet graft in the future.

## **Acknowledgements**

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## **CHAPTER 4**

### **Local Expression of Indoleamine 2,3 Dioxygenase in Fibroblasts Significantly Prolongs Survival of an Engineered 3D Islet Allograft<sup>3</sup>**

#### **4.1 Introduction**

Insulin-producing islet cells are selectively and irreversibly destroyed in type 1 diabetes resulting in insulin deficiency with severe acute and chronic complications. Given the improvements in organ preservation and islet isolation techniques, endocrine replacement therapy by islet transplantation represents a feasible and attractive alternative therapeutic approach for treating patients with type 1 diabetes (1, 2). Despite improvement in the duration of allogeneic islet engraftment using systemic immunosuppression, islet transplantation is still limited by high rates of rejection. Furthermore, some of the immunosuppressive agents are pro-diabetogenic and associated with hyperlipidemia, hypertension, nephrotoxicity, neurotoxicity and increased risk of malignancy (3-6). Finding more efficient and less harmful strategies to protect islet grafts are therefore required for improving islet transplantation outcome.

Localized expression of immunoregulatory factors using gene transfer to the graft is a feasible method to provide an immunoprivileged microenvironment and consequently improve graft survival. Such an on-site delivery system results in more potent local immunosuppression with less systemic side effects. In fact, local expression of immunosuppressive agents has been demonstrated in many transplant models to block the immune response to the graft (7-9).

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<sup>3</sup> A version of this chapter has been submitted for publication. Jalili RB, Forouzandeh F, Moeenrezakhanlou A, Medina A, Larijani B, Ghahary A. Local Expression of Indoleamine 2,3 Dioxygenase in Syngeneic Fibroblasts Significantly Prolongs Survival of an Engineered 3D Islets Allograft.



IDO is a cytosolic enzyme that catalyzes the conversion of essential amino acid L-tryptophan to kynurenine (10) and has profound effects on T-cell proliferation, differentiation, effector functions and viability (11). Both the reduction in local tryptophan concentration and the production of immunomodulatory tryptophan metabolites contribute to the immunosuppressive effects of IDO (12, 13). Broad evidence implicates IDO and the tryptophan catabolic pathway in generation of immune tolerance to antigens in tissue microenvironments. In particular, the role of IDO in fetal tolerance in mammalian pregnancy (14, 15), immunologic tolerance to tumors (16, 17), and self tolerance (18, 19) has been documented. The unique immunoregulatory function of IDO substantiates the application of this enzyme as a strategy to suppress alloimmune responses in transplantation.

Our research group provided compelling evidence in support of the fact that the expression of IDO in bystander fibroblasts suppresses immune response and improves outcome of skin grafts (20-25). With the aim of using IDO immunosuppressive activity in islet transplantation setting, in another study we were able to confirm that bystander IDO-expressing fibroblasts suppressed immune response to allogeneic mouse islets *in vitro* (26). Furthermore, in a recent study we showed that mouse islets and fibroblasts are selectively resistant to IDO-mediated activation of nutrient deficiency stress pathway and that coculture of mouse islets in a three-dimensional IDO-expressing fibroblast populated collagen matrix does not have any deleterious effect on viability and insulin secretory capacity of islets *in vitro* and *in vivo* (27). Here, we engineered a 3D composite islet allograft equipped with IDO-expressing fibroblasts and examined whether local expression of IDO, conferred by adenoviral-mediated gene transfer to bystander syngeneic fibroblasts, prevents the rejection of islet allograft. In this study we showed that local IDO expression significantly improved

islet allograft survival, prevented T-cell infiltration to the graft and delayed alloantibody production as long as IDO transgene was expressed in the graft. Our approach here is novel compared to other studies that examined the suppressive effect of IDO in islet transplantation (28, 29) because a) bystander syngeneic fibroblasts were used as the target of gene transfer instead of islets to avoid deleterious effects of adenovirus infection on islets (30-32), b) islets were embedded within an extracellular matrix which by itself improves islet function and viability (33, 34) and c) co-transplanted fibroblasts are more than just a source of IDO and can enhance islet physiological competence (35, 36).

## **4.2 Materials and Methods**

### **Mouse Islet isolation**

Islets were obtained from 6 to 8-week-old male BALB/c mice (The Jackson Laboratories, Bar Harbor, ME, USA) as previously described (26). Briefly, mice were anesthetized and pancreases were distended through the pancreatic duct with 2.5 ml of Hanks' balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD) containing 2.0 mg/ml of collagenase (Type V; Sigma Chemical Co., St Louis, MO). The distended pancreases were then removed and incubated at 37°C for 15 min. The islets were purified by discontinuous centrifugation on Ficoll (Sigma) gradients. After centrifugation, islets were handpicked and cultured in HAM's F10 medium (Sigma) supplemented with 12 mmol/l HEPES, 2 mmol/l L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in 95% air, 5% CO<sub>2</sub> at 37°C. Care and maintenance of all animals were in accordance with the principals of laboratory animal care and the guidelines of institutional Animal Policy and Welfare Committee.

### **Preparation of 3D islet-fibroblast composite grafts**

Mouse dermal fibroblasts were explanted from B6 mice skin and transduced with a recombinant adenoviral vector carrying human IDO cDNA as described previously (26). Control fibroblasts were infected with a mock vector or left untreated. Fibroblast populated collagen gel (FPCG) matrices were prepared as described by Sarkhosh et al. (23) using IDO-expressing or control fibroblasts. Mouse islets were added to FPCG before solidification in 24 well plates. The composite grafts were maintained in 95% air, 5% CO<sub>2</sub> at 37°C until transplantation time.

### **Transplantation of islet- fibroblast composite grafts**

Recipient B6 mice were rendered diabetic by a single intraperitoneal injection of 200 mg/kg streptozotocin (Sigma) and diabetes was defined as a minimum of two consecutive blood glucose measurements  $\geq 20$  mmol/l. Islet plus IDO-expressing or control fibroblast composite grafts (approximately 500 islets) were transplanted under the left kidney capsule of isoflurane-anesthetized diabetic mice. After transplantation, blood from the tail vein of each recipient was collected two times a week between 7:00 and 9:00 AM to determine the normalization of blood glucose levels. Blood glucose levels were measured using an Accu-Chek Compact Plus blood glucose monitoring system (Roche Diagnostics, Laval, QC, Canada) and grafts were deemed functioning when blood glucose levels decreased to  $< 180$  mg/dl. All animals were cared for according to the guidelines of the institutional Animal Policy and Welfare Committee.

### **Intraperitoneal glucose tolerance test**

An intraperitoneal glucose tolerance test (IPGTT) was performed in mice transplanted with IDO expressing composite grafts two and four weeks after transplantation. After a 16-h

overnight fast, glucose (2 mg/g body wt) was injected intraperitoneally into nonanesthetized mice. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120 min. Area under the curve (AUC) was determined using SigmaPlot software (Systat Software Inc., San Jose, CA)

### **Histological analyses**

Graft bearing kidneys were harvested at indicated time points, fixed in 10% buffered formalin solution and embedded in paraffin. Sections 5  $\mu$ m thick from graft area were stained with hematoxylin and eosin to show graft morphology. To detect insulin-containing beta-cells and determine the infiltration of T-cells into the grafts, double immunofluorescence for insulin and CD3 was performed on 5  $\mu$ m thick sections from paraffin embedded samples. These sections were rehydrated and then nonspecific binding was eliminated by incubating the tissue sections in 5% goat serum (Sigma) and 2% fetal bovine serum (Sigma) in PBS for 60 min. Sections were then incubated overnight at 4°C with guinea pig anti-insulin antibody (1:500 dilution, Dako Laboratories, Mississauga, ON, Canada) and rabbit anti-CD3 antibody (1:100 dilution, abcam, Cambridge, MA). After three washing steps with TBS-Tween 20 for 5 minutes each, samples were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG (1:2000 dilution, abcam) and rhodamine- conjugated anti-rabbit IgG (1:2000 dilution, Chemicon International, Temecula, CA) for 45 minutes in dark.

To test the IDO transgene expression in the composite grafts, IDO immunofluorescence staining was performed on a separate set of graft sections. The procedure for staining was as described above and the primary and secondary antibodies used were rabbit anti-IDO Ab (1:1000 dilution, raised in rabbit by Washington Biotechnology Inc. Baltimore, MD) and rhodamine- conjugated anti-rabbit IgG (1:2000 dilution, Chemicon), respectively. Finally,

after washing with TBS-Tween 20 three times for 5 minutes each, samples were mounted in Vectashield H-1200 (Vector Laboratories Inc., Burlingame, CA) containing 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining. A Zeiss Axioplan 2 microscope and Northern Eclipse image analysis software were used to obtain the images.

### **IDO transgene quantitative PCR**

Total RNA was isolated from harvested grafts at indicated time points using RNeasy kit (Qiagen, Maryland). cDNA was synthesized using SuperScript first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA ). Briefly, 2 µg of total RNA was reverse transcribed in a 20-µl reaction volume for 50 min at 42°C with SuperScript First-Strand Synthesis System for RT-PCR and oligo dT15 primer, according to the manufacturer's protocol. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in a reaction mixture of 25 µl using the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) PCR kit according to the manufacturer's instructions. The reactions were run on a 7900 HT Fast Real time PCR system (Applied biosystems, Foster City, CA). Human IDO-specific mRNA was quantified relative to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the following primers: *IDO* forward, 5'-CCTGAGGAGCTACCATCTGC-3' reverse, 5'-TTGGCTTCCAGTTTTGAAGG-3'; *GAPDH* forward, 5'-GGCATTGCTCTCAATGACAA-3' reverse, 5'-TGTGAGGGAGATGCTCAGTG-3'. All the measures were performed in duplicate. The analyses were performed with the Sequence Detection system 7900HT software version 2.3 (Applied biosystems). GAPDH was used to adjust IDO mRNA expression. The ratio of IDO to GAPDH was calculated according to the formula: ratio =  $2^{-dCt}$  (dCT = mean Ct gene -

mean Ct housekeeping). The results were expressed as the percentage of IDO transgene mRNA expression at the indicated time point to that of day one post-transplantation.

### **IDO western blotting, RT-PCR and kynurenine assay**

Fibroblasts were harvested and lysed 72 hours post infection with IDO or mock adenovector. For western blot analysis, equal amounts of cell lysate were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The blots were then probed with polyclonal anti IDO antibody raised in rabbit by Washington Biotechnology Inc. (Baltimore, MD) at a final dilution of 1:5000. Horseradish peroxidase conjugated goat anti-rabbit IgG served as a secondary antibody for the enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Santa Cruz, CA).

For IDO reverse transcriptase (RT)-PCR, total RNA was isolated using RNeasy kit (Qiagen, Maryland). cDNA was synthesized using SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Primers were as described for quantitative PCR methods above. Amplified PCR products were separated by 2% agarose gel electrophoresis and visualized with SYBER Safe DNA gel staining (Invitrogen).

The level of kynurenine was measured as previously described (26) . Briefly, proteins in conditioned medium were precipitated by trichloroacetic acid. After centrifugation, 0.5 ml of supernatant was incubated with equal volume of Ehrlich's reagent for 10 minutes at room temperature. Absorption of resultant solution was measured at 490 nm by spectrophotometer.

### **Serum alloantibody detection**

Blood serum samples were collected from composite graft recipients B6 mice (H-2<sup>b</sup>) at indicated time points post-transplantation. Donor BALB/c mice (H-2<sup>d</sup>) thymocytes ( $1 \times 10^6$ ) were incubated with recipient B6 mouse serum at 1:128 dilution for 1 h at 37 °C

(5% CO<sub>2</sub>, 95% air). Thymocytes were then washed with PBS and incubated with Alexa Flour 488 goat anti-mouse IgG (1:200 dilution; Invitrogen) for 1 h at 4 °C. Binding of cells to antibody was detected from single-parameter fluorescence histograms using a BD FACSCanto flow cytometer (BD Biosciences, Mississauga ON, Canada) after gating on viable cells. Controls for this experiment include sera from non-transplanted naïve B6 mice and thymocytes stained with secondary antibody alone without mouse serum.

### **Statistical analyses**

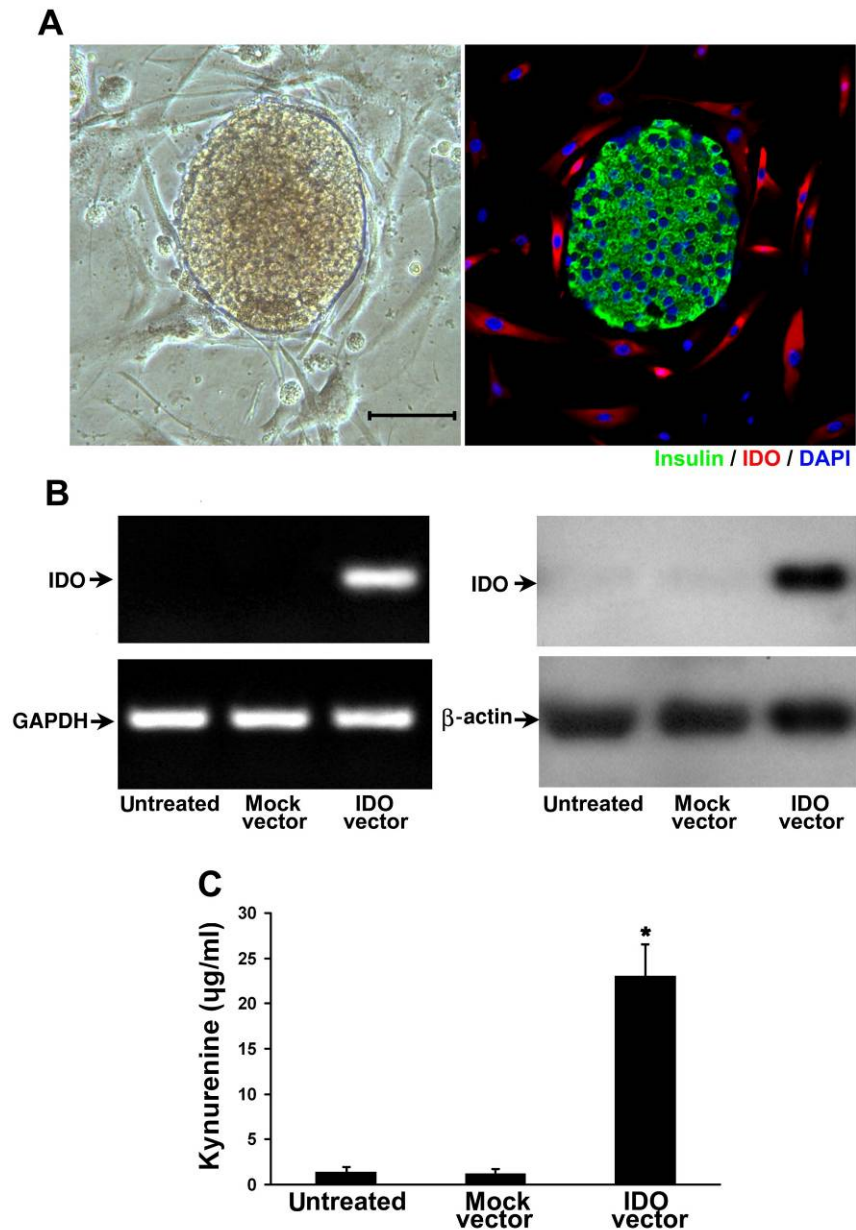
Data are reported as mean  $\pm$  standard error of mean (SEM) of three or more independent sets of experiments. Survival of islet grafts in IDO-expressing versus control groups were compared using Kaplan-Meier log-rank test. The statistical differences of mean kynurenine and IDO mRNA levels among treated and control groups were tested with one-way ANOVA. Post hoc comparisons were done using Student's *t*-test with Bonferroni correction for multiple comparisons. Statistical analyses were performed with SPSS statistical software, version 13.0 for Windows (Chicago, IL). *P*-values less than 0.05 were considered statistically significant.

## **4.3 Results**

### **Morphology of composite islet graft and IDO expression by fibroblasts in the graft.**

Mouse fibroblasts were induced to express IDO using an adenoviral vector as described in the Methods. Mock virus infected and uninfected fibroblasts were used as controls. Three-dimensional grafts were then engineered by embedding mouse islets within IDO-expressing or control fibroblast populated collagen matrix. The figure 4.1 shows morphology of composite graft and IDO expression by fibroblast in the graft. As shown in

the figure 4.1A, islets were surrounded by fibroblasts within the collagen matrix. Double immunofluorescence staining for IDO and insulin confirmed IDO expression in the fibroblasts that enfolded islets (Fig. 4.1A, right panel). RT-PCR and western blot analysis confirmed IDO expression in adenoviral transduced fibroblasts at mRNA (Fig 4.1B, left panel) and protein (Fig. 4.1B, right panel) levels, respectively. Measurement of kynurenine (tryptophan metabolite) in the graft conditioned media further confirmed enzymatic activity of IDO (Fig. 4.1C). These findings demonstrate that in the engineered 3D composite grafts, insulin producing islets are covered with fibroblasts which express high levels of IDO.





**Figure 4.1.** Microscopic appearance of composite islet graft and IDO expression in fibroblasts. Mouse islets were embedded within IDO-expressing or control fibroblast populated collagen matrix. (A) Photomicrograph of composite graft under inverted microscope (left panel) shows the structure of the graft. Double immunofluorescence staining for IDO (red) and insulin (green) confirms IDO expression in fibroblasts surrounding an islet(right panel).(B) RT-PCR (left panel) and western blotting (right panel) for IDO in untreated, mock virus infected and IDO virus infected fibroblasts. GAPDH and  $\beta$ -actin were used as loading control for RT-PCR and western blotting, respectively. (C) Kynurenine levels in conditioned media of composite grafts after 72 hours of culture. Asterisk highlights statistically significant difference ( $p < 0.001$ ). Error bars indicate standard error of the mean (SEM). Scale bar: 100  $\mu$ m

### **Local expression of IDO prolongs islet allograft survival and function.**

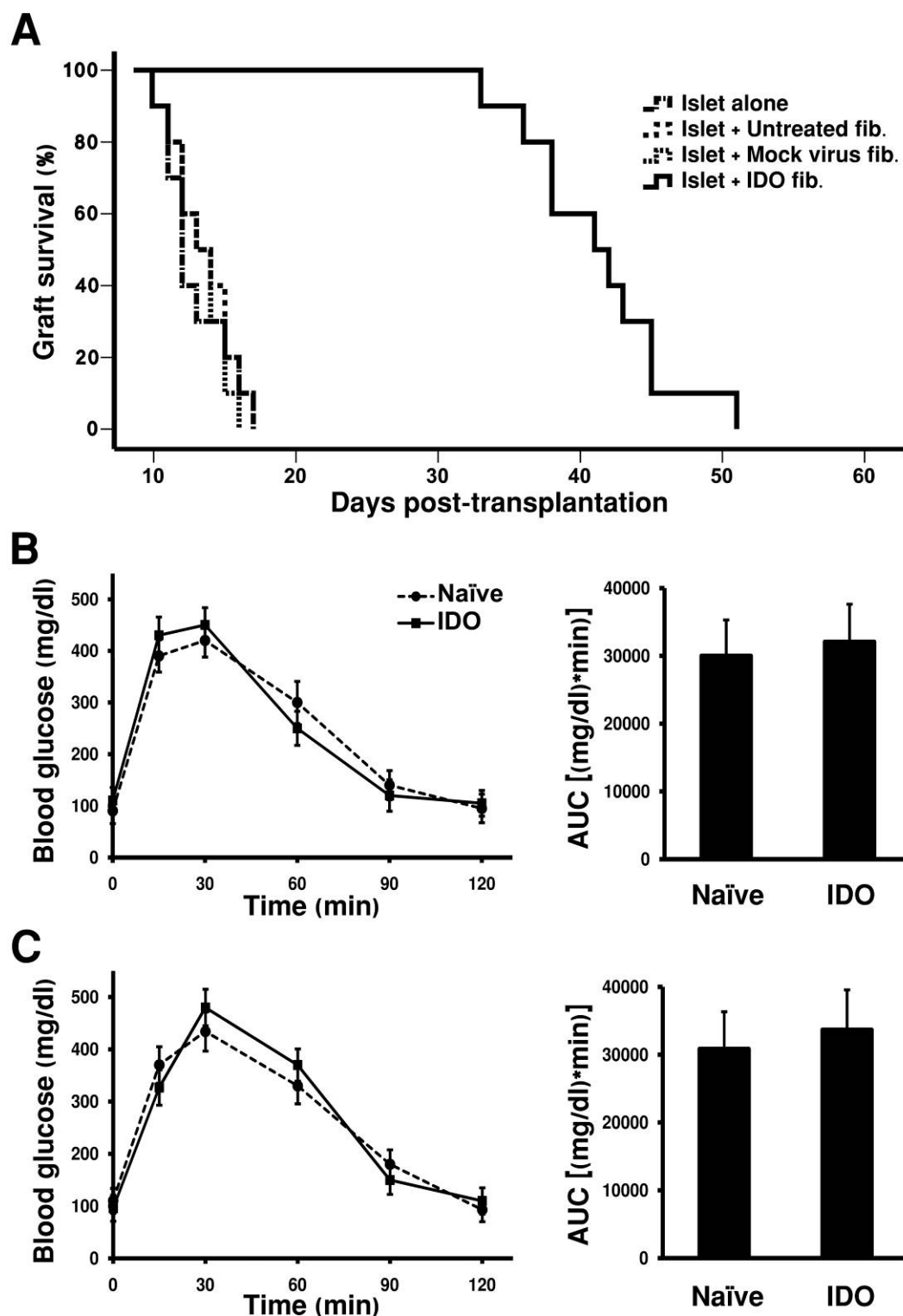
To investigate the local immunosuppressive effect of IDO, 3D grafts were engineered by embedding 500 BALB/c mouse islets within collagen matrix populated with IDO-expressing or control B6 mouse fibroblasts. These composite grafts were then transplanted to renal subcapsular space of streptozotocin-induced diabetic immunocompetent B6 mice. Another control group of mice received only islets. Islet graft function was checked by measuring blood glucose in graft recipient mice. Composite IDO-expressing grafts showed a significant prolongation of graft survival ( $41.2 \pm 1.64$  days,  $p < 0.001$ ,  $n=10$ ; table and Fig. 4.2A). In contrast, as shown in the table and the figure 4.2A, control grafts were rejected within 2 weeks after transplantation. Mean duration of graft survival in the islet-alone group and the grafts with untreated and mock virus treated fibroblast were  $12.9 \pm 0.73$ ,  $13.5 \pm 0.79$ , and  $13.2 \pm 0.61$  days, respectively (table). The mean survival duration for mock vector infected composite grafts was not significantly different from those of grafts with untreated fibroblasts and islet-alone grafts. This confirmed that adenoviral infection by itself had no influence on graft survival.

**Table. 4-1** Composite islet graft survival in different experimental groups

<b>Group</b>	<b><i>n</i></b>	<b>Graft survival (days post-transplantation)</b>	<b>Mean <math>\pm</math> SEM</b>	<b>p value <sup>A</sup></b>
Islet + Untreated fib.	10	10, 11, 12( $\times$ 2), 13, 14, 15 ( $\times$ 2), 16, 17	13.5 $\pm$ 0.79	0.706
Islet + Mock vector fib.	10	10, 11, 12( $\times$ 2), 13, 14( $\times$ 2), 15( $\times$ 2), 16	13.2 $\pm$ 0.61	0.908
Islet + IDO fib.	10	33, 36, 38( $\times$ 2), 41, 42, 43, 45( $\times$ 2), 51	41.2 $\pm$ 1.64	< 0.001
Islet alone	10	10, 11( $\times$ 2), 12( $\times$ 3), 13, 15, 16, 17	12.9 $\pm$ 0.73	-

<sup>A</sup> p value for difference in mean survival durations compared with islet alone group calculated using Log Rank (Mantel-Cox) test.

To further confirm normal function of the IDO-expressing islet allografts, intraperitoneal glucose tolerance test (IPGTT) was performed on the mice that received IDO-expressing composite grafts after two and four weeks post-transplantation. As shown in the figures 4.2B and 4.2C the glucose clearance rate in mice received IDO-expressing grafts was similar to naïve animals after two weeks (AUC: 32078  $\pm$  5550 vs. 30002  $\pm$  5300 (mg/dl)\*min, n=3, p>0.05) and four weeks (AUC: 33673  $\pm$  5900 vs. 30836  $\pm$  5500 (mg/dl)\*min, n=3, p>0.05) post-transplantation, respectively. The normal response of islet grafts to IPGTT demonstrated that islets in the IDO-expressing composite grafts are able to function normally in response to glucose load, suggesting preservation of islet mass. These data collectively confirm that local IDO expression significantly prolongs islet allograft survival.

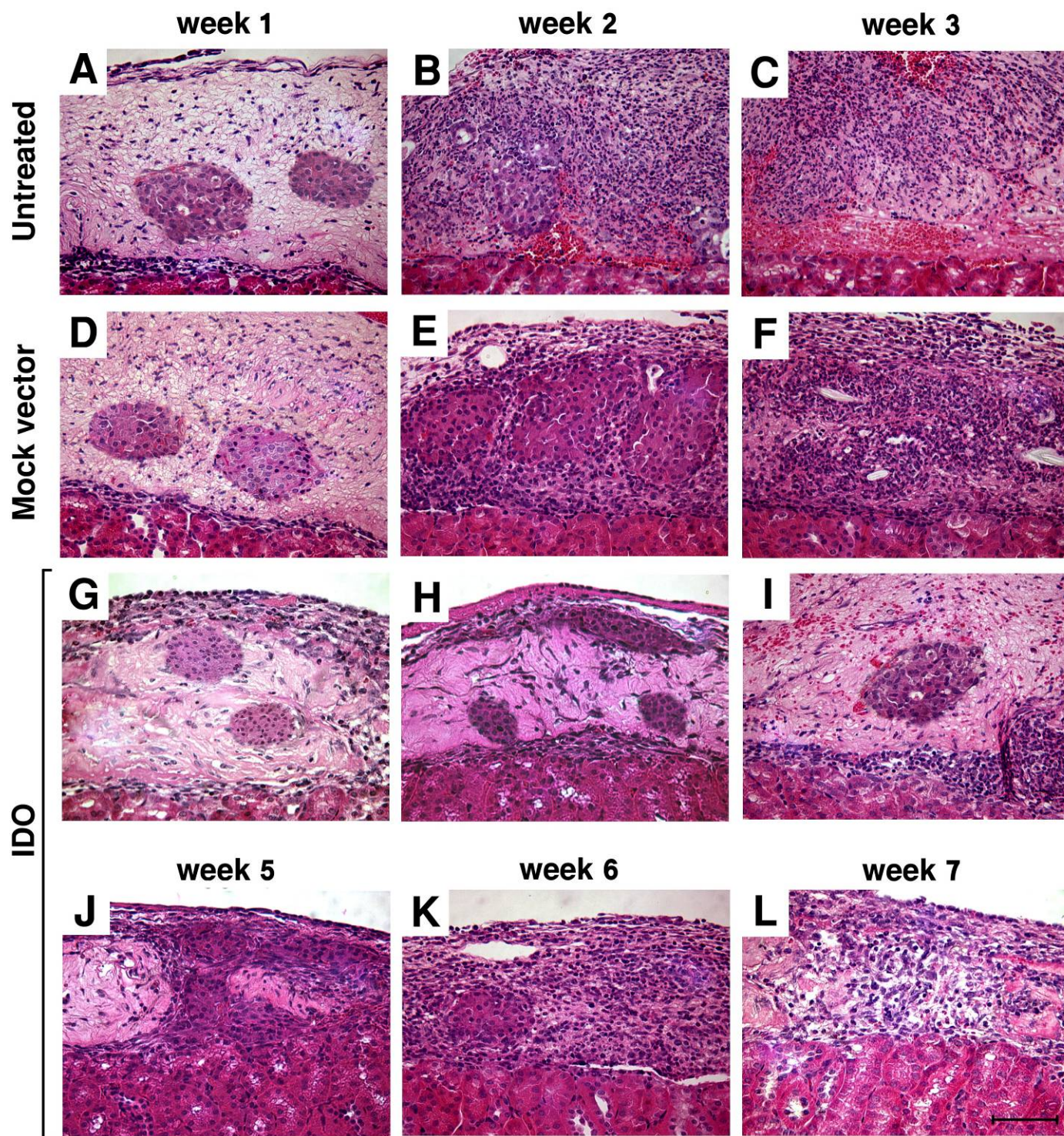


**Figure 4.2.** Islet graft survival and function after transplantation. (A) Kaplan-Meier survival curve shows prolongation of IDO-expressing grafts survival (solid line) compared to islet alone (dash-dot line), untreated (dashed line), and mock virus infected (dotted line) grafts (n=10). Intraperitoneal glucose tolerance tests (IPGTT) after two weeks (B) and four weeks (C) post-transplantation confirmed normal glucose responsiveness in graft bearing mice (solid line) versus naïve mice (dashed line) (n=3). Bar charts on the right panels show area under the IPGTT curves. Error bars indicate standard error of the mean (SEM).

### **IDO prevents infiltration of lymphocytes into composite grafts.**

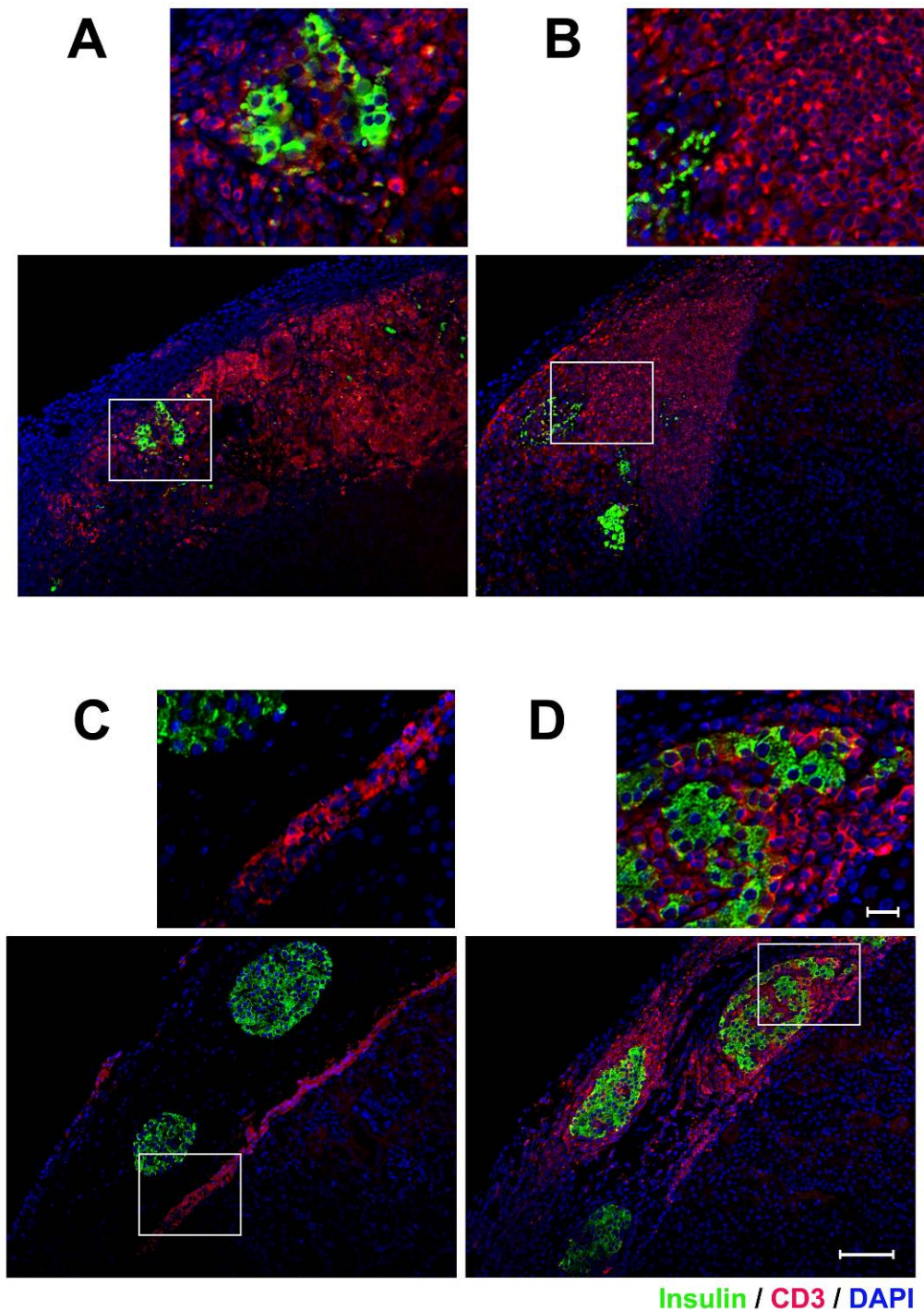
To examine the histopathological changes in islet allografts, a group of graft recipient mice were euthanized at the end of each week post-transplantation. Composite grafts were then recovered and stained with H&E or subjected to double immunofluorescence staining for CD3 and insulin. Histological studies demonstrated that islets architecture was well preserved in the IDO-expressing composite grafts for up to 6 weeks (Fig. 4.3G-L) whereas islets in the untreated (Fig. 4.3A-C) or mock vector infected grafts (Fig. 4.3D-F) were almost completely destroyed before the third week post-transplantation. Control grafts were diffusely infiltrated with mononuclear cells as early as second week post-transplantation (Fig. 4.3B and E) while IDO-expressing grafts remained intact for up to 6 weeks (Fig. 4.3G-K). Furthermore, double staining for CD3 and insulin clearly showed very few insulin producing cells and dense T-cell infiltration in the both untreated (Fig. 4.4A) and mock vector infected controls (Fig. 4.4B) at the end of the second week post-transplantation. In contrast, islets in the IDO-expressing grafts were strongly stained for insulin and maintained their normal architecture with minimal infiltration of T-cells at the same time point (Fig. 4.4C). It has to be emphasized that T-cells densely accumulated in the border of IDO-expressing graft and kidney tissue but didn't penetrate the composite graft (Figs. 4.3I and 4C). However, T-cells started to infiltrate IDO vector infected grafts by the end of the fifth week post-transplantation (Fig. 4.4D). Taken together, these data suggest that local expression of IDO prevents islet graft rejection via suppression of lymphocytes and prevention of T-cell infiltration into islet allograft.





**Figure 4.3.** Histology of composite islet grafts. Graft recipient mice were euthanized at indicated time points post-transplantation. Composite islet grafts were then retrieved and stained with H&E. (A-C) Untreated and (D-F) mock vector infected fibroblast grafts after 1, 2, and 3 weeks post-transplantation, respectively. (G-L) IDO-expressing fibroblast grafts after 1-3 and 5-7 weeks post-transplantation. Note inflammation and cellular infiltration into the graft started in control groups in the second week but in IDO group delayed until 6th week post-transplantation. Scale bar: 100  $\mu$ m

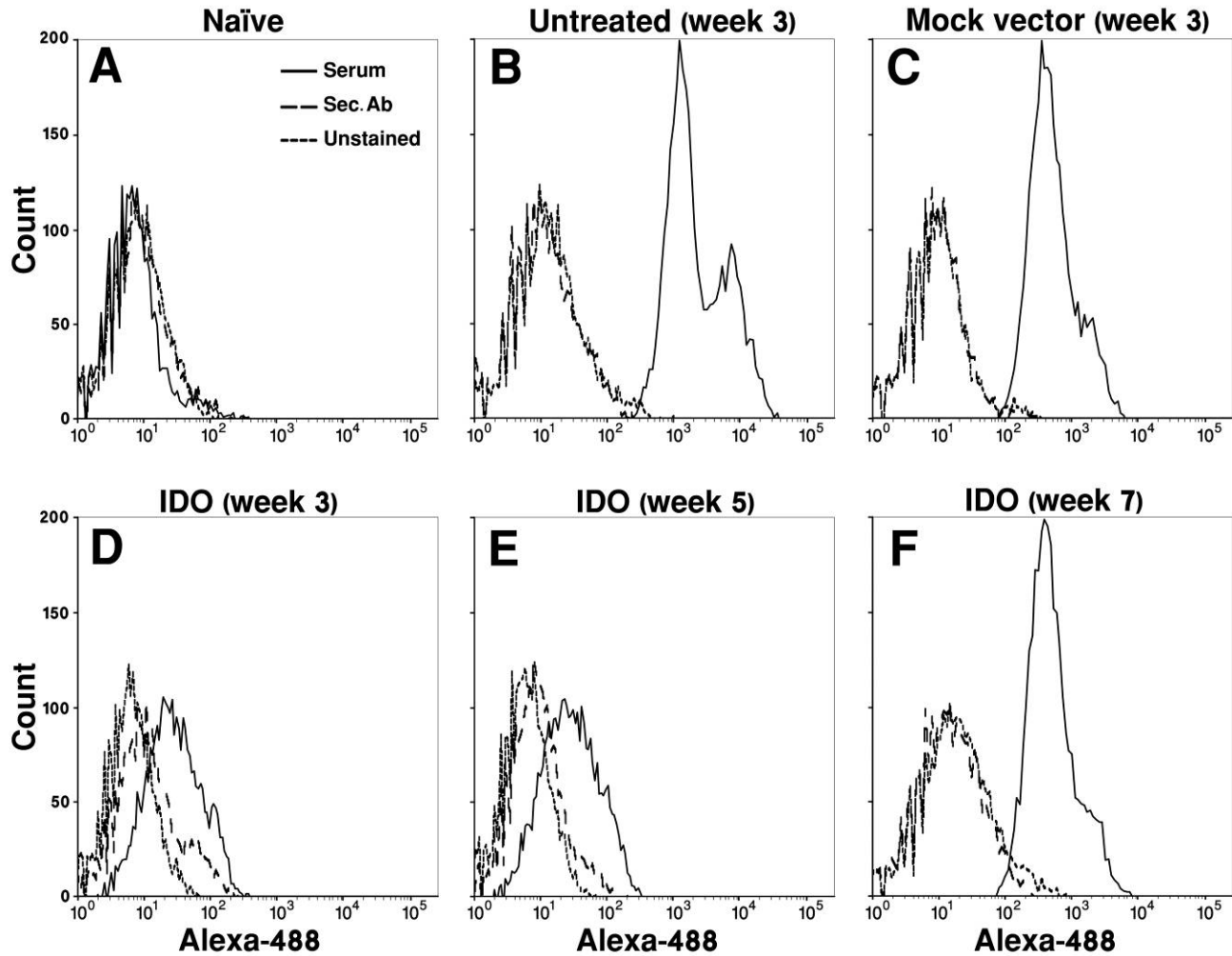




**Figure 4.4.** Lymphocyte infiltration into composite islet grafts. Graft recipient mice were euthanized at indicated time points post-transplantation. Composite islet grafts were then retrieved and subjected to double immunofluorescence staining for CD3 (red) and insulin (green). (A-C) composite grafts in untreated, mock vector infected and IDO-expressing fibroblast grafts at two weeks post-transplantation. Upper smaller panels show high magnification of the indicated area of the lower panels. Note in the IDO-expressing graft, CD3<sup>+</sup> cells accumulated in the border of the graft and kidney tissue but didn't infiltrate the graft. (D) IDO vector infected graft after 5 weeks post-transplantation. Scale bars in the low and high magnification panels equal to 100 and 20 μm, respectively.

**Production of donor specific alloantibodies is delayed in IDO-expressing graft recipients.**

To further investigate the mechanism of immunosuppression by IDO, we examined the production of donor specific alloantibodies in composite graft recipients. Sera from graft recipient animals were tested for presence of antibodies against donor antigens using FACS analysis as described in the Methods. As shown in the figure 4.5, when donor mouse thymocytes (H-2<sup>d</sup>) were incubated with sera from B6 mice (H-2<sup>b</sup>) three weeks after receiving composite grafts consisting of untreated or mock vector infected fibroblasts, majority of thymocytes were bound with alloreactive mouse IgG antibodies which indicated development of high levels of anti donor antibodies (Fig. 4.5B and C). In contrast, sera from IDO-expressing graft recipients showed very low levels of donor-specific antibodies until seventh week after transplantation (Fig. 4.5D-F). This finding suggests that suppression of humoral immune response is probably one of the mechanisms by which IDO prevents allograft rejection.



**Figure 4.5.** Donor specific antibody production in graft recipient mice. BALB/c thymocytes (H2d) were incubated with serum collected from naïve B6mice (H2b) (A), mice received graft with untreated (B), mock vector infected (C), and IDO expressing (D) fibroblasts after three weeks post-transplantation. For IDO vector infected group the experiment repeated at weeks 5 (E) and 7 (F) post-transplantation. Binding of donor specific antibodies (solid line) was measured and analyzed by flow cytometry. Controls for this experiment consisted of unstained cells (dotted line) and secondary antibody without serum (dashed line). Note production of donor specific antibodies in IDO expressing group occurred four weeks later than control groups.



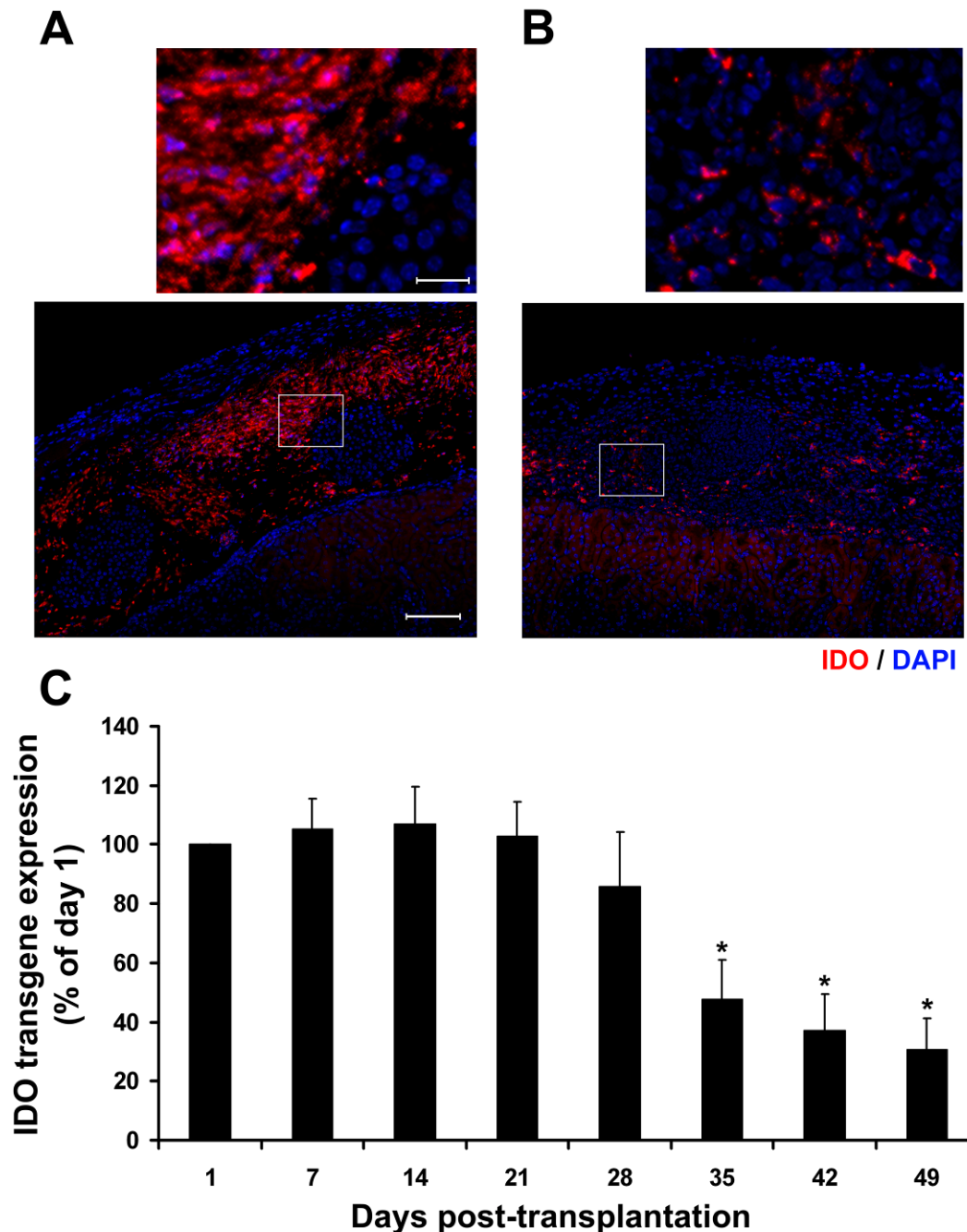
### **Duration of IDO transgene expression in composite grafts corresponds to graft survival length.**

In view of limited length of anti rejection effect of IDO in this study, we hypothesized that a decline in the level of IDO transgene expression can be considered as the possible cause of late graft rejection in IDO vector infected group. We therefore tested the length of intra-graft IDO transgene expression using IDO immunofluorescence staining and quantitative real-time PCR. A group of IDO composite graft recipient animals were euthanatized at the end of each week post-transplantation. Composite grafts were then retrieved and were subjected to IDO immunofluorescence staining or qPCR. As shown in the figure 4.6A, IDO protein (red) is expressed uniformly and widely in fibroblasts throughout the graft when tested two weeks after transplantation. However, after four weeks only a limited number of cells in the graft were still positive for IDO protein (Fig. 4.6B).

For analyzing quantitative PCR results, adjusted levels of IDO mRNA at each time point were calculated as the percentage of IDO mRNA expression on the day one post-transplantation. The results showed that IDO transgene was expressed at high levels in composite grafts containing IDO vector infected fibroblasts for up to four weeks post-transplantation and then the expression level started to decrease. As presented in the figure 4.6C, significant decreases in the intra-graft IDO transgene expression were observed from 100% on day one to  $47.6 \pm 13.4$ ,  $37.2 \pm 12.3$ , and  $30.6 \pm 10.7$  on days 35, 42, and 49 post-transplantation, respectively ( $p < 0.001$ ,  $n=3$ ). This finding was consistent with *in vitro* lasting effect of IDO expression in fibroblasts following adenoviral IDO transduction (data not shown). IDO transgene mRNA was not found in the control grafts (data not shown). As the IDO transgene was from human origin, by using human specific primers we expected that

intrinsic IDO expression (i.e. mouse IDO that expressed in grafts as a result of inflammation) did not interfere with IDO transgene qPCR.

These data collectively show a transient pattern for IDO transgene expression in composite grafts following adenoviral gene transfer. The time course of intra-graft IDO expression closely corresponds to the duration of graft survival and also to cellular and humoral alloimmune responses timeline. We therefore suggest that late graft rejection in this study was probably due to time dependent loss of transient IDO expression. As such, IDO immunosuppressive effect and islet graft survival can be significantly extended by application of stably transduced IDO-expressing bystander fibroblasts.



**Figure 4.6.** Stability analysis of IDO transgene expression in composite islet grafts. IDO graft recipient mice were euthanized at indicated time points post-transplantation. Composite islet grafts were then retrieved and subjected to immunofluorescence staining or quantitative RT-PCR for IDO. (A and B) immunofluorescence staining of IDO protein (red) in IDO vector infected grafts after two weeks (A) and five weeks (B) post-transplantation. Upper smaller panels show high magnification of the indicated area of the lower panels. Scale bars in the low and high magnification panels equal to 100 and 20  $\mu$ m, respectively. (C) IDO transgene mRNA levels measured by quantitative RT-PCR in IDO vector infected grafts on days one to 49 post-transplantation. The level of IDO mRNA at each time point was normalized as the percentage of IDO mRNA level on day one post-transplantation. Asterisks highlight statistically significant difference compared to IDO mRNA level on day one post-transplantation ( $n=3$ ,  $p<0.001$ ). Error bars indicate standard error of the mean (SEM).

## 4.4 Discussion

In the present study, we showed that local IDO expression prevents cellular and humoral alloimmune responses against islets and significantly prolongs islet allograft survival without systemic anti rejection treatments in diabetic immunocompetent mice. IDO plays a crucial role in suppression of immune responses. Several previous studies implicated immunomodulatory effect of IDO in different settings including islet transplantation (28, 29, 37). However, this is for the first time that IDO-expressing bystander syngeneic fibroblast populated collagen scaffold is used to protect islet allograft. The engineered composite graft that was developed and transplanted in this study has several unique features. First, we avoided to directly transfer the IDO transgene to islets to reduce the risk of cytotoxicity and loss of islet function. There are reports showing that adenoviral gene transfer to islets interferes with beta cell function and increases apoptosis rate (32, 38) which is not even counteracted by Bcl-2 overexpression (30). Potiron et al showed that islet graft transduction with adenoviruses coding for costimulation inhibitors was associated with high level of primary islet nonfunction and was unable to protect islet xenografts from rejection (31). Furthermore, it was shown that multiple chemokines and chemokine receptors are expressed by murine islets in response to adenovirus transduction and it impairs engraftment of marginal mass of transplanted islets (39). As another limitation for adenoviral gene transfer to islets, gene expression after transduction is observed only in the periphery of islets but not in the islet core (40). To achieve an efficient adenoviral gene transfer to the pancreatic beta cells, monolayer formation of the islets is critical (41). Therefore, by using bystander fibroblasts and not islets as the target for IDO gene transfer, we were able to induce high

levels of IDO expression as a local immunosuppressive factor while avoiding any deleterious consequence of gene transfer on islet survival and function.

The second advantageous characteristic of this composite graft is embedment of islets within a 3D extracellular matrix (ECM). ECM is one of the most important constituents of the islet microenvironment. Several studies demonstrated that entrapment of islets within a collagen matrix resulted in satisfactory morphology, enhanced viability, and improved insulin secretory capability in islets (42-44). As such, embedding islets within ECM, by itself, can improve islet graft function. In fact, embedding mouse islets with a 3D collagen scaffold can reduce the critical islet mass required for achievement of normoglycemia in a murine model although it cannot prevent immunological rejection (unpublished data). Moreover, as shown in the figure 4.1, collagen matrix provides a supportive scaffold and helps IDO-expressing fibroblasts surround and protect islets very efficiently in a 3D structure.

Co-transplantation of fibroblasts is another beneficial aspect of this composite graft. Fibroblasts are cells of choice to be used as bystander IDO-expressing cells because a) they can be easily induced to express IDO, b) show low levels of DNA synthesis and become quiescent when embedded in a collagen matrix (45) and c) are resistant to apoptosis and IDO induced low tryptophan environment (25, 27). Fibroblasts can also improve islet cell viability. The essential role of fibroblasts in islet physiological competence was previously reported and it was shown that some fibroblast produced factors can promote islet survival in culture (35, 36). In addition, availability of syngeneic or autologous fibroblasts eliminates the risk of immune response against these cells. Collectively, application of this novel composite islet graft can address a number of common obstacles in islet allotransplantation by

preventing immunological rejection, reestablishing islet cell-ECM interaction, and probably providing essential growth factors required for islet survival and function.

Findings of the present study suggest that T-cell suppressive effect of IDO is the major mechanism of its anti rejection activity. As clearly shown in the figures 4.3 and 4.4, T-cells accumulated at the margins of the IDO-expressing grafts but did not invade and infiltrate islets. This accords with the very well known function of IDO that is generation of a low tryptophan and high kynurenine microenvironment within which activated T-cells are not able to proliferate and survive. Data presented in the figure 4.2 determine that such a microenvironment protects islet grafts rejection but does not negatively affect islet function.

This study for the first time showed that local IDO expression can inhibit production of donor specific alloantibodies. The mechanism of this inhibition needs further elucidation. It is well documented that humoral response against donor-specific antigens is clearly dependent on help provided by CD4<sup>+</sup> T-cells (46). Only CD4<sup>+</sup> T-cells recognizing alloantigen through the indirect pathway (i.e. CD4 T-cells recognize processed allogeneic MHC presented as peptide in the context of recipient MHC class II) are able to provide cognate help to allospecific B-cells for the development of alloantibody (47). As such, inhibition of alloantibody production in our study might be due to T-cell suppressive effect of IDO which significantly reduces the number of donor-specific T-cells and consequently results in insufficient cognate help to B-cells.

Finite survival of islet grafts in this study may appear discouraging in the first place. However, the data presented here clearly show that as long as high levels of intra-graft IDO expression was maintained, islet grafts survived and functioned normally and cellular and humoral immune response against islets were suppressed very efficiently. This finding

suggests that long-term protection of islet grafts is feasible if stable IDO expression in bystander fibroblasts achieved.

In conclusion, the present study proves the feasibility of development of a viable and functional IDO-expressing composite islet graft and confirms that IDO efficiently and significantly improves islet allograft survival. This promising finding proves the local islet-directed immunosuppressive effect of IDO and sets the stage for development of a non-rejectable islet allograft using stable IDO induction in bystander fibroblasts. This approach is currently under our investigation.

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## CHAPTER 5

### Conclusion and Suggestions for Future Work

#### 5.1 General Discussion and Conclusion

Although advances in insulin therapy modalities have improved management of type 1 diabetes (T1D) very significantly, these treatments neither cure diabetes nor completely prevent its complications. The replacement of insulin producing tissue by pancreatic islet transplantation offers a physiological approach for precise restoration of glucose metabolism in patients with T1D. Over more than two decades, significant advances have been made in different aspects of islet transplantation (1-6). Reports of both short-term and long-term insulin-independence and improvement in recipients' quality of life following human islet allotransplantation (7-13) have encouraged several centers in the world to continue clinical trials of islet transplantation in patients with T1D. However, several obstacles remain to be overcome before the procedure could be proposed to a larger patient population, a prominent one of which is toxicity and inadequate efficiency of current immunosuppressive regimens used after transplantation. The ultimate goal of islet transplantation is therefore to completely reverse diabetic state without the need for lifelong immunosuppressive drug therapy.

Unlike solid organs, it is feasible to manipulate and modify islets *ex vivo* before transplantation. In view of this unique opportunity, many attempts have been made to develop innovative strategies for immunological conditioning of islet to maintain long-term graft survival without immunosuppressive agents (14-22). However, unfortunately none of these studies resulted in development of a practical islet transplantation model so far.

To address this challenging problem, in this thesis research we developed and applied a novel composite islet graft equipped with a potent immunomodulatory factor, IDO.

Engineering of this composite graft was rationalized based on the fact that IDO plays an essential protective role during mammalian pregnancy with providing a low tryptophan / high kynurenine microenvironment in which maternal T-cell cannot proliferate and attack semi-allogeneic fetus. This thesis involved several studies which focused on local immunosuppressive effect and safety of IDO in an islet transplantation model. As noted in Chapter 2, we were able to confirm that local expression of IDO in bystander cells suppress *in vitro* islet allogeneic response very efficiently. In fact, IDO expression resulted in almost five-time reduction in lymphocyte proliferation rate compared to the control (figure 2.5, Chapter 2). Furthermore, as discussed in Chapter 3, we showed that islets survived and functioned normally within this composite IDO-expressing graft using several experimental approaches including islet viability and apoptosis assays, measurement of  $\beta$ -cell insulin content, and islet glucose responsiveness test. More importantly, it was shown that the IDO-expressing islet grafts were viable and functional for more than 100 days when transplanted to a syngeneic diabetic animal (Fig 3.6, Chapter 3). These data confirmed safety of IDO-induced low tryptophan and high kynurenine microenvironment for islet survival and physiology. In the final phase of this thesis, to test the immunosuppressive effect of IDO within islet allografts, the composite islet grafts were transplanted into renal subcapsular space of immunocompetent chemically-induced diabetic mice. As presented in Chapter 4, IDO expressing grafts survived significantly longer than controls. Allogeneic islets remained intact and functional in IDO-expressing grafts without receiving systemic immunosuppressive agents for more than six weeks. Local expression of IDO clearly prevented lymphocyte infiltration into islet grafts. While T-cells densely accumulated in the border of IDO-expressing grafts and kidney tissue, they didn't invade the composite grafts

(Fig 4.4, Chapter 4). These findings collectively confirmed that development of a functional IDO-expressing composite islet graft is feasible and local expression of IDO in bystander cells effectively protects islet allografts.

There are several important aspects that distinguish this thesis from previous studies. First, we targeted bystander fibroblasts for IDO gene transfer instead of islets. This approach prevents possible cytotoxic effect of adenovector-mediated gene transfer to islets (23, 24). Moreover, in this method, in contrast to direct islet transduction in which transgene expression is usually limited to the superficial layer of cells (25), high levels of transgene expression is expected because theoretically all bystander cells can be infected with the vector. Additionally, when islets are directly transduced, as IDO is a cytosolic and not a secretory protein, the boundary of low tryptophan microenvironment will be very close to the first line of transplanted islets. This may let infiltrating T-cells come to close contact with these islets and possibly attack some of them before going through apoptosis.

Second improvement in this composite graft is application a collagen matrix as scaffold. As discussed in Chapter 3, entrapment of islets within a collagen matrix per se improves islet viability and function *in vitro*. And finally bystander fibroblasts embedded within the matrix maintain the integrity of the composite graft and support islets possibly by production of growth factors (26, 27).

Two findings of this thesis are reported here for the first time. First, in Chapter 3 we showed that IDO activates nutrient deficiency stress kinase general control nonderepressible 2 (GCN2) and its downstream signaling pathway, selectively and specifically in lymphocytes but not in islet cells. This is an important piece of evidence because activation of GCN2

pathway is a known mechanism for IDO induced apoptosis (28). As such, this finding further confirms the differential suppressive effect of IDO on immune cells and its safety for islets.

The second new finding of this study, as presented in Chapter 4, was the alloantibody hyporesponsiveness found in IDO-expressing islet graft recipients. The effect of IDO on T-cells and cellular immune response is very well established but to our knowledge this is for the first time that effect of IDO on humoral immune response is reported. How local IDO expression affects humoral immune system is a very intriguing question and the mechanism(s) of this phenomenon needs further elucidation.

In summary, in this thesis research we were able to 1) confirm islet immunosuppressive effect of IDO, 2) demonstrate that development of a viable and functional composite islet allograft consisting of islets embedded within IDO-expressing fibroblast populated collagen matrix is feasible, and 3) apply this novel composite islet graft in an allergenic diabetic mouse model and show IDO-mediated prolonged graft immunoprotections.

## **5.2 Suggestions for Future Work**

Although we believe that the findings of this thesis research are very promising, we obviously just past the proof-of-principle phase and this idea needs further improvement. The followings are my suggested studies to be done as next steps for improvement of this non-rejectable islet graft model.

- 1- As in Chapter 4 we showed that immunosuppressive effect of IDO is expected as long as high levels of IDO is expressed in the graft, we hypothesize that stable IDO expression in bystander fibroblast will result in long-term islet graft survival. As such

using appropriate stable gene transfer strategies (e.g. application of a lentiviral vector) is suggested to extend the graft survival.

- 2- The animal model used in this thesis was designed to address alloimmune response to islet grafts. However islet grafts in type 1 diabetes face both allo and autoimmune destruction. As such, we suggest testing IDO immunosuppressive function in an autoimmune diabetes model such as NOD mice. We are optimistic that IDO may be effective in suppressing islet autoimmune attack as it has been shown that tryptophan metabolism is defective and IDO expression in response to IFN- $\gamma$  is absent in NOD mouse (29, 30). Thus, restoring IDO function through its local expression in islet graft may result in suppression of autoimmune response.
- 3- As the ultimate goal for development of the non-rejectable islet graft is its application in a clinical setting, we suggest working on safer non-viral methods for IDO gene transfer to fibroblasts. This approach will help the model be more translatable to the clinic.
- 4- As IDO, depending on the condition, can function as both an immunosuppressive and a tolerogenic factor, we suggest studying the immunological mechanism(s) underpinning IDO anti rejection function in more detail to clarify the possibility of induction of tolerance by using local IDO expression.
- 5- Finally we suggest further investigating the two new findings of this thesis that is selective GCN2 pathway activation in immune versus non immune cells and IDO-mediated suppression of humoral immunity.



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THE UNIVERSITY OF BRITISH COLUMBIA

## ANIMAL CARE CERTIFICATE

**Application Number:** A06-1474

**Investigator or Course Director:** Aziz Ghahary

**Department:** Surgery

**Animals:**

Mice C57BL/6 144  
Mice NOD 113  
Mice BALB/c 451

**Start Date:** February 1, 2007

**Approval Date:** January 21, 2009

**Funding Sources:**

**Funding Agency:** Canadian Institutes of Health Research (CIHR)

**Funding Title:** Development and application of a non-rejectable engraftment of allogeneic pancreatic islets populated collagen-GAG gel in diabetic animal model

**Unfunded title:** N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

**A copy of this certificate must be displayed in your animal facility.**

Office of Research Services and Administration  
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3  
Phone: 604-827-5111 Fax: 604-822-5093

## Appendix II



The University of British Columbia



### Biohazard Approval Certificate

PROTOCOL NUMBER: <b>H05-0103</b>
INVESTIGATOR OR COURSE DIRECTOR: <b>Ghahary, Aziz</b>
DEPARTMENT: <b>Surgery</b>
PROJECT OR COURSE TITLE: <b>Development of non-rejectable allogeneic pancreatic islets</b>
APPROVAL DATE: <b>05-12-05</b>
APPROVED CONTAINMENT LEVEL: <b>2</b>
FUNDING AGENCY: <b>Canadian Institutes of Health Research</b>

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

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Approval of the UBC Biohazards Committee by one of:  
Chair, Biosafety Committee  
Manager, Biosafety Ethics  
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

**A copy of this certificate must be displayed in your facility.**

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